

Identification and Characterization of novel *Fasciola Hepatica* Sodium Chloride dependent Taurine Transporter

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Submitted by

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Declaration

Herewith I declare that the experiments of the present thesis were carried out under the supervision of Dr. Oliver Kudlacek and Prof. Dr. Michael Freissmuth.

The cloning of YFP-Fh TauT was done by Sandra Pichler whereas I generated the non-tagged version of FhTauT. The western blot in the manuscript was generated with the help of Martina Molin. Uptake experiments carried out with the supervision and the support of Marion Holy. The experiments in this thesis were mainly performed by me.



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Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CFP	Cyan fluorescent protein
Cl⁻	Chloride ion
C-terminus	Carboxyl-terminus
DMEM	Dulbecco's modified Eagle Serum
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
Fh	Fasciola hepatica
GABA	γ -Aminobutyric acid
GAT1	γ -Aminobutyric acid transporter 1
GES	Guanidinoethyl sulphonate
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HEK	Human embryonic kidney cells
HsTauT	Homo sapiens taurine transporter
kDa	Kilo dalton
KHP	Krebs-HEPES buffer
K_m	Michaelis-Menten constant
MDR	Multi drug resistance

mRNA	messenger RNA
Na⁺	Sodium ion
NBD	Nucleotide binding domain
nH	Hill coefficient
N-terminus	Amino-terminus
PBS	Phosphate buffered saline
PDL	Poly-D-lysine
P-gP	P-glycoprotein
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
SBD	Substrate binding domain
SDS PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis
SERT	Serotonin transporter
SLC6	Solute carrier 6
TauT	Taurine transporter
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline included Tween-20
TCBZ	Triclabendazole
TCBZ.SO	Triclabendazole sulfone
TM	Transmembrane
TMD	Transmembrane domain
V_{max}	Maximal velocity
YFP	Yellow fluorescent protein

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Abstract

The economic burden imposed by the liver fluke *Fasciola hepatica* is the result of its infestation of domestic ruminants. The fact that several million people worldwide are infested by this trematode has led the World Health Organization (WHO) to classify human fascioliasis as a disease of vital global public health importance. Because a vaccine against this parasite is not available anthelmintic drugs are the only option, for both animals and people. As an anthelmintic chemotherapeutic, triclabendazole has been the drug of choice in fighting this parasite for more than 25 years. Triclabendazole is active against both adult and juvenile flukes and is well tolerated by the host organisms. However, the increasing prevalence of flukes resistant to triclabendazole has made the identification of alternative drug targets a priority.

In this study, I focused on the fact that adult *F. hepatica* persists in the hostile environment of the bile ducts of infected organisms. In mammals, bile acids are rendered less toxic by conjugation to taurine (2-aminoethanesulfonic acid). This reaction is thus also contingent on cellular uptake of taurine. In my thesis, I cloned the *Fasciola hepatica* taurine transporter, a member of the solute carrier-6 (SLC6) family. A comparative analysis of the sequence indicated its close relation to unknown SLC6 transporters from related nematodes (i.e., *Clonorchis sinensis* and *Schistosoma* spp.) analysis When heterologously expressed in HEK293 cells, this *F. hepatica* transporter supported the high-affinity cellular uptake of taurine ($K_M=12.0 \pm 0.5 \mu\text{M}$) but not of GABA. Substrate uptake was dependent on Na^+ and Cl^- (calculated stoichiometry 2:1). Consistent with the low chloride concentration in mammalian bile, the *F. hepatica* transporter had a higher apparent affinity for Cl^- ($\text{EC}_{50}=14\pm3 \text{ mM}$) than the human taurine transporter ($\text{EC}_{50}=55\pm7 \text{ mM}$). I incubated flukes with unconjugated bile acids in the presence and absence of taurine and found that taurine promoted the survival of flukes, while the taurine

transporter inhibitor guanidinoethansulfonic acid abolished this protective effect of taurine. These observations support the conclusion that the taurine transporter is critical for the survival of liver flukes in the bile. Thus, the taurine transporter represents a viable drug target candidate.



Zusammenfassung

Die wirtschaftliche Belastung durch den Leberegel *Fasciola hepatica* resultiert aus dem Befall von Wiederkäuern, die als Nutztiere gehalten werden. Die Tatsache, dass mehrere Millionen Menschen weltweit von diesem Trematoden befallen sind, hat die Weltgesundheitsorganisation (WHO) dazu veranlasst, die menschliche Fascioliasis als eine Krankheit von globaler Bedeutung für die öffentliche Gesundheit zu klassifizieren. Da keine effektive Impfung gegen diesen Parasiten existiert, beruht die Behandlung von Tieren und Menschen nur auf Anthelminthika. Von diesen ist Triclabendazol seit mehr als 25 Jahren das Mittel der Wahl zur Bekämpfung dieses Parasiten. Triclabendazol wirkt sowohl gegen adulte als auch juvenile Egel und ist gut verträglich. Das zunehmende Auftreten von Triclabendazol-resistenten Egel macht die Suche nach alternativen Ansatzpunkten erforderlich. Adulte *Egel* persistieren in den Gallengängen des Wirtsorganismus, wo sie dem aggressiven Milieu der Galle ausgesetzt sind. Gallensäuren werden im Normalfall mit Taurin (2-Aminoethansulfonsäure) konjugiert, wodurch sie weniger toxisch werden. In der vorliegenden Arbeit wurde Transporter aus der solute carrier-6 (SLC6) -Familie kloniert, der homolog zum menschlichen Taurintransporter ist. Die phylogenetische Analyse zeigte, dass dieser Transporter am nächsten mit dem GABA-Transporter-2 von Invertebraten und unklassifizierten Transportern anderer Nematoden verwandt ist. Nach heterologer Expression in HEK 293 Zellen, vermittelte dieser *F. hepatica*-Transporter eine hochaffine zelluläre Aufnahme von Taurin ($K_M = 12,0 \pm 0,5 \mu\text{M}$), nicht jedoch von GABA. Die Substrataufnahme war abhängig von Na^+ - und Cl^- (berechnete Stöchiometrie 2: 1). Übereinstimmend mit einer relativ geringen Chloridkonzentration in der Galle von Säugertieren zeigte der *F. hepatica* Transporter eine offensichtlich höhere Affinität für Cl^- ($\text{EC}_{50} = 14 \pm 3 \text{ mM}$) als der menschliche Taurintransporter ($\text{EC}_{50} = 55 \pm 7 \text{ mM}$). Experimentell konnte gezeigt werden, dass unkonjugierte Gallensäuren rasch zum Tod von *Fasciola hepatica* führen. Die Zugabe von Taurin ermöglichte hingegen das Überleben der Egel in Gegenwart von Gallensäuren.

Guanidinoethansulfonsäure, ein Inhibitor des Taurintransporters, hob diese schützende Wirkung von Taurin wieder auf. Diese Beobachtungen Untersuchungen legen den Schluss nahe, dass der Taurintransporter für das Überleben von Leberegeln in der Galle essentiell ist. Somit stellt der Taurintransporter einen möglichen Angriffspunkt für neue anthelminthische Wirkstoffe dar.



1. General Introduction

1.1. *Fasciola hepatica*

Liver flukes are parasitic flatworms (Platyhelminthes: Trematoda: Digenea) that have a socioeconomic impact on livestock animals and humans in several countries. The two representatives of *Fasciola* genera are *Fasciola gigantica* and *Fasciola hepatica* (Latin, fasciola = small bandage; Gr., hepar = liver). *Fasciola hepatica* is the form predominantly found in temperate climate zones, while *Fasciola gigantica* infests people and livestock in the tropics of Africa and Asia. *F. hepatica* is about 3.5 cm long and 1.5 mm wide (**Figure 1**), whereas *Fasciola gigantica* is about twice as large reaching 7.5 cm in body length. Compared to other digeneans, *F. hepatica* has equal size of ventral and oral suckers; extremely branched testes and intestinal caeca; a small, intricately folded uterus; and vitellaria that goes beyond to the posterior end together with the lateral edges of the body.

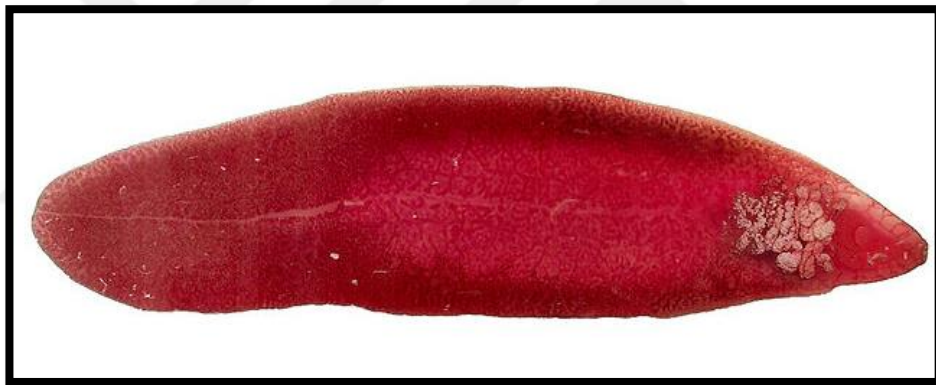


Figure 1. An image of a liver fluke (*Fasciola hepatica*; trematode). The figure is taken from Wikimedia Commons, the free media repository. (<https://commons.wikimedia.org>).

1.2. Life Cycle of *Fasciola Hepatica*

F. hepatica, like other members of the genera *Opisthorchis* and *Clonorchis*, resides in liver tissues and connecting organs such as the bile duct and gallbladder of their mammalian hosts. After maturation (about 12 weeks), flukes lay their eggs (dimensions: 130-150 μm by 63-90 μm), which are dispersed via the feces of the host. To continue the life cycle, the eggs must reach fresh water,

where they hatch after up to 2 weeks (in fresh water at around 22°C): the miracidium leaves the egg through operculum and uses its eyespots to locate its intermediate host, a freshwater snail, which it penetrates, within 24 hours of hatching. After penetration, each miracidium develops into a sporocyst giving rise to mother rediae, which, consecutively, produce daughter rediae in the snail's digestive gland. After the daughter rediae develop into cercariae, cercariae leave the snail and become free-swimming organisms. These encyst on grass and other plants to form metacercariae: this stage is ingested, by both livestock animals and people. In the small intestines of the definitive host, the metacercariae develop into juvenile flukes (this is also known as the acute phase). The juvenile flukes penetrate the gut wall to reach the liver via the abdominal cavity. They move through liver tissues until they reach the bile duct (also known as the chronic phase) (**Figure 2**). An adult *F. hepatica* can live several years in the host (Durbin, 1952).

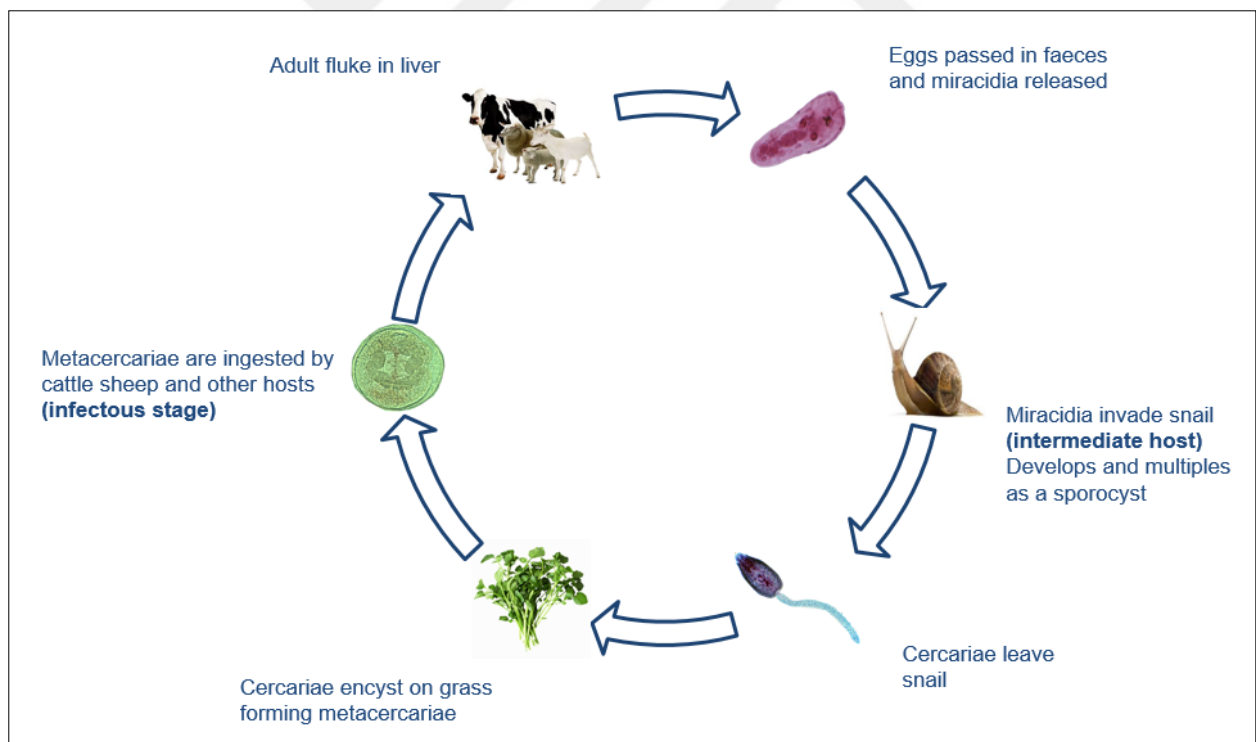


Figure 2. The life cycle of *Fasciola hepatica*. For details see text.

1.3. Epidemiology

The permanent hosts in nature are typically ruminant herbivores. People are infested accidentally by ingestion of metacercariae encysted on plants. In addition, people are at risk, if they ingest water containing metacercariae. In fact, this can give rise to regional pockets of endemic infections, e.g., on the Bolivian Altiplano (Villegas *et al*, 2012). Accordingly, fascioliasis is also relevant to human health: conservative estimates indicate that more than 2.5 million people are infested and suffer from various forms of fascioliasis (Fürst *et al*, 2012). Fascioliasis does not affect one specific risk group: it can affect people from all age-groups. Although people living in rural communities are more prone to infestations, fascioliasis may occur anywhere and in any country, where people are consuming carrier plants as a part of their diet. Fascioliasis has been recognized as a neglected tropical disease, but it is also seen as a global threat because human infestations have been reported from more than 75 countries worldwide (WHO) (**Figure 3**).

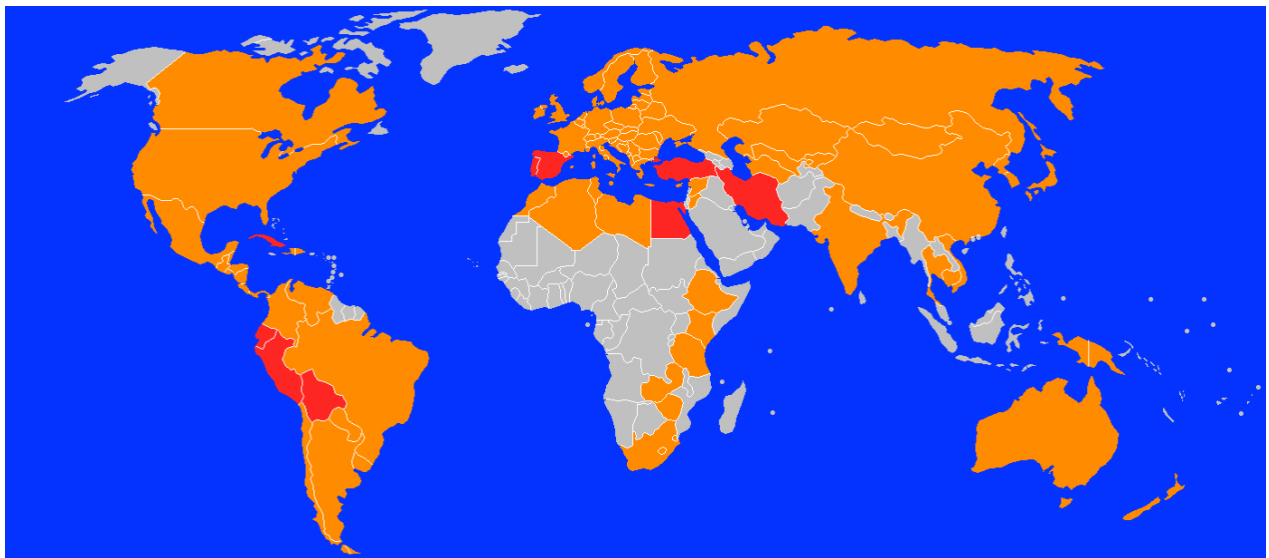


Figure 3. Fasciola hepatica prevalence. The countries in red are those with high incidence, those in orange demonstrating have low-medium incidence (The image is taken from https://en.wikipedia.org/wiki/Fasciola_hepatica).

1.4. Pathogenesis and Clinical Signs

Behm and Sangster (1999) divided the clinical course of fascioliasis into two phases: the parenchymal phase and biliary phase. In the parenchymal stage, juvenile flukes penetrate the intestinal wall, gain access to the abdominal cavity, where they move along the curvature of the abdominal wall until they find the liver and invade the liver parenchyma. In the biliary phase, juvenile flukes complete their development into adult flukes in the bile ducts. The level of infection is correlated with the clinical signs that indicate the severity of the disease. Only about 15% of the fascioliasis cases are symptomatic, whereas the rest of the cases stays asymptomatic (Fürst *et al*, 2012).

According to Mas-Coma et al. (2014), the clinical course can be divided into four phases.

1.4.1. Incubation period

This phase starts with the ingestion of metacercariae and can last for days or even months. This time is based merely on the number of ingested metacercaria and the health status of the patient or the infested animals.

1.4.2. Acute Fascioliasis

At this stage, several juvenile fluke larvae start to migrate from the place of peritoneal cavity to the area of biliary tree and liver. This stage can last about 2 to 4 months. Due to the destruction of peritoneum and hepatic tissue, human patients show either localized or generalized allergic and toxic reactions. These symptoms include fever, generalized abdominal pain or pain in the right upper quadrant of the abdomen, loss of appetite, nausea, hepatomegaly, flatulence, and diarrhea, shortness of breath, chest pain, cough, and urticaria.

In case of animal infestation, the migration of larvae may result in liver failure and intraperitoneal hemorrhage (Behm & Sangster, 1999). This stage often causes weight loss, anemia, diarrhea and in some cases resulting in enlargement of the liver. It has already been reported that acute fasciolosis

may cause sudden death in sheep and is responsible of losses of up to 25% of the animals within flocks (Behm & Sangster, 1999). However, in the majority of cases, the infested animals do not show any apparent symptoms at this stage.

1.4.3. Latent Phase (subacute fascioliasis)

In this stage of the disease, maturation occurs, and eggs are released into the bile duct. Infested animals often develop hemorrhagic anemia with this type of fascioliasis. Human patients suffer from gastrointestinal disorders; laboratory investigations reveal eosinophilia.

1.4.4. Chronic Fascioliasis

At this stage, several symptoms can be observed in affected animals including paleness, weight loss, bottle jaw, and break in wool in sheep (Garcia *et al*, 2008; McKenna *et al*, 2002; Roberts *et al*, 2006; Behm & Sangster, 1999). The function of liver of infested animals can be impaired leaving them more susceptible to secondary bacterial infections, e.g. by *Clostridium novyi*, resulting in a condition known as Black's disease (Jensen & Swift, 1982). Such condition makes animals more sensitive to abortions and reduces milk production in lactating animals. The loss of animal only occurs when chronic fascioliasis seriously weakens the animal .

The degree of infestation is correlated with the clinical signs, which also give the severity of the disease. In humans, only about 15% of the fascioliasis cases are symptomatic, whereas the rest of the cases stays asymptomatic (Fürst *et al.*, 2012).

1.5. Treatment of Fascioliasis

1.5.1. Diagnosis

The destruction of liver parenchyma results in the release of cellular enzymes (e.g., aspartate aminotransferase/AST, glutamate dehydrogenase, γ -glutamyl transpeptidase), which can be detected in plasma. This elevation of circulating enzymes, however, is non-specific. Similarly, the destruction

of liver parenchyma and the resulting reactions (perilesional edema, abscess formation, thickening of the bile duct, and so forth) can be visualized by computer tomography and by sonography (Kabaalioglu *et al*, 2007; Teke *et al*, 2014). In some instances, the flukes have been directly visualized by computer tomography (Kabaalioglu *et al*, 2007) and by ultrasonography (Teke *et al*, 2014). Confirmation of the diagnosis, however, required the detection of anti-fluke antibodies. The antibodies can be detected by enzyme-linked immunosorbent assay (ELISA) in blood (serum or plasma) using the excretory-secretory antigens or recombinant *F. hepatica* cathepsin-L1, the immunodominant component thereof, as capture antigen. The ELISA has been adapted in various formats to afford the detection of antibodies in milk because this allows for monitoring the infestation of cattle and sheep in the field (Reichel, 2002). However, the most commonly used method is the detection of characteristic eggs in the feces of the patients (Ward *et al*, 1997) (**Figure 4**). There are different methods for investigating eggs such as using individual or composite samples, flotation, and sedimentation. However, fecal egg counts (FECs) alone is not seen as an indication of fluke numbers because of irregular egg shedding (Anderson *et al*, 1999; Flanagan *et al*, 2011).

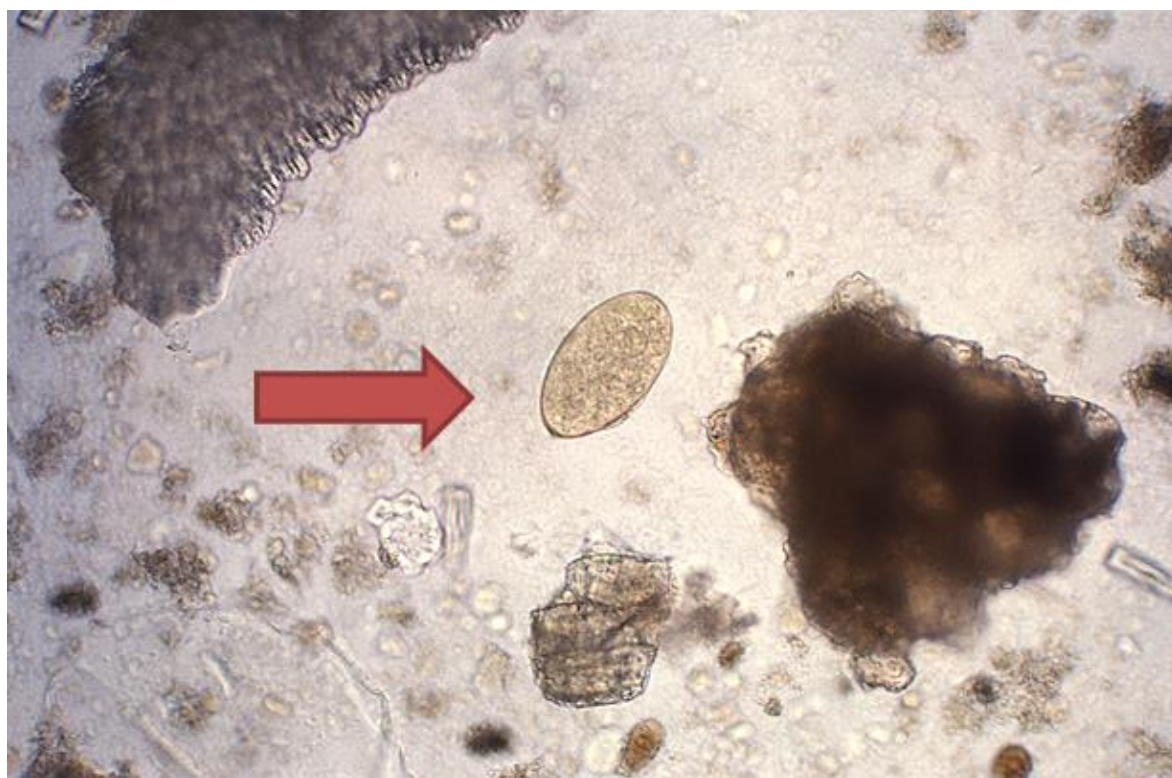


Figure 4. Fasciola hepatica egg. A *F. hepatica* egg is visualized in an unstained wet mount. The image is taken from (<https://www.cdc.gov/parasites/fasciola/diagnosis.html>) This image shows a typical *F. hepatica* egg (red arrow) under the magnification of 128X, which is distinguished as broadly ellipsoidal, operculated. The unfertilized eggs pass in feces. The figure is provided by Dr. Mae Melvin which is available online at Public Health Image Library (PHIL) with the ID#: 1540. (<https://phil.cdc.gov/>)

The determination of antibodies by ELISA is more sensitive than fecal egg counts because it can detect fluke infections much earlier than fecal egg counts (Salimi-Bejestani *et al*, 2005). However, antibodies persist after elimination of parasites. Accordingly, the specificity of anti fluke antibodies is lower than fecal egg counts for the diagnosis of an active infestation.

1.5.2. Drugs

Although alternative drugs are available, triclabendazole (TCBZ) has been the drug of choice for the treatment of fascioliasis for more than 25 years because of its high efficacy against both adult and juvenile flukes (Table 1). It is the only drug recommended by World Health Organization (WHO), and its cure rate is high while side effects are temporary and mild. Both in clinical practice and preventive chemotherapy interventions, the recommended dose is 10 mg/kg body weight, and it is administered as a single dose. The dosage might be increased to 20 mg/kg body weight in two separate doses in clinical practices.

The mechanism of the drug is assumed to bind the microtubulin proteins of a fluke (using an analogy to other benzimidazoles). The research on tegument, vitelline and testis support this assumption (Fairweather, 2005). Flukes treated with TCBZ.SO also leads to a decrease of tubulin immunoreactivity (McConville *et al*, 2006).

Anthelmintic	Flukicide Activity
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	1-6 weeks	6-12 weeks	Adult
Albendazole	X	X	✓
Clorsulon	X	X	✓
Closantel	X	✓	✓
Nitroxynil	X	✓	✓
Oxyclozanide	X	X	✓
Triclabendazole	✓	✓	✓

Table 1. Efficacy of flukicides available for use in cattle. (The table is adapted from Fairweather & Boray, 1999))

1.5.3. The host immune system

To date, the immune response of hosts to *F. hepatica* has still not been fully appreciated. *F. hepatica* elicits a Th2 biased response, which has been shown to be important in the elimination of particular parasites. In the initial stages of infection, Th1 cells are also considered to play a role (Rodríguez-Sosa *et al*, 2002). In the course of the migratory phase of the newly excysted juvenile (NEJ) and immature fluke, Th1 cells produce INF- γ , which activates macrophages. These macrophages engage the parasite and produce nitric-oxide (Noël *et al*, 2004), which is known as toxic to the fluke (Moreau & Chauvin, 2010). At the stage of chronic fascioliasis, the immune response becomes mainly Th2 biased.

Flukes have many mechanisms for evading the immune system; thus, they can remain alive for an extended period within their host (Fairweather & Boray, 1999). However, it is not well understood, how the parasite evades or modulates the immune system. The final place in the bile duct is an

immunologically safe environment because the parasite is not readily accessible to the by the immune system of the body (Hanna *et al*, 1982). However, it must escape the immune response while migrating through the intestinal wall and liver tissue. Only 5 to 10% and 20 to 25% of the inocula in cattle or sheep, respectively, reach maturity in experimental infections, showing that a significant amount of the newly excysted juvenile flukes are not able to enter the gut or killed during the migrating phase (Piacenza *et al*, 1999). As a defense mechanism, the tegument of *F. hepatica* plays a vital role in the evasion of immune attacks by renewing both glycoproteins and the glycocalyx (Tkalcevic *et al*, 1995; Hanna, 1980). Besides, releasing immunosuppressive factors helps flukes to modulate the immune system of the definitive host (Sandeman & Howell, 1981; Zimmerman *et al*, 1983).

1.5.4. Vaccination

Vaccination of animals would undoubtedly be a wise alternative to flukicides because residual chemicals impose waiting periods on the marketing of milk and meat. This results in economic losses. In addition, repeated exposure to anthelmintic drugs increases the risk for the emergence of drug resistance (Overend & Bowen, 1995). A commercial vaccine is currently not available for prevention of fascioliasis (see review Toet *et al*. 2014). Yet, immunoprophylactic control of fasciolosis has been tried in both cattle and sheep by injecting parasite extracts or defined parasite antigens that differ in the efficacy level, which they elicit. These antigens include cathepsin L-like cysteine proteases, leucine aminopeptidases (LAP), fatty acid binding protein (FABP), fluke hemoglobin and glutathione S-transferases (GSTs) (Spithill & Dalton, 1998). Here, I will focus on glutathione S-transferases (GSTs) as representative antigens.

1.5.5. Glutathione S-transferases

Of particular interest, concerning both drug resistance studies and vaccine trials are the glutathione transferases (GSTs) (EC 2.5.1.18). As a family of phase II enzymes, the glutathione transferases (GSTs) are ubiquitously expressed in both eukaryotes and prokaryotes. These enzymes are best known for their ability to conjugate glutathione (GSH) to potentially toxic substrates. The resulting biotransformation leads to detoxification. These substrates are either endogenous products formed during oxidative stress or exogenous compounds (xenobiotics) (Hayes & Pulford, 1995). Alterations in the expression levels of GST-isoforms or their subcellular distribution can have significant biological and clinical consequences (Kodym *et al*, 2001; Lombardi *et al*, 2015; Piaggi *et al*, 2010).

F. hepatica can survive for more than ten years within the biliary tract, where free oxygen radicals are common due to lipid peroxidation and several hydrophobic substances produced by liver metabolism (Lechner *et al*, 2002). To adapt to the hostile environment, *F. hepatica* continuously produces detoxification enzymes, and among them, glutathione transferases are the major components. Until now, at least five different types of GST isozymes (mu, sigma, omega, microsomal, zeta) have been identified. Some of them have been proven to be promising vaccine candidates (Morrison *et al*, 1996; Spithill *et al*, 1997).

Due to the success rate at closely-related *Schistosoma* spp., GST proteins were considered as candidate vaccine antigens. The reduction of parasite burden in mice was ranging from 40 to 43 %; whereas for rats it was more successful; from 50 to 72 %, which were injected with two vaccination doses of GST before a subsequent challenge with 15,000 cercariae (Balloul *et al*, 1987; Brophy & Pritchard, 1994). At the beginning, vaccination with native GSTs were unsuccessful to reduce worm burdens in rats, which were infected with *F. hepatica* experimentally (Howell *et al*, 1988), although the next trial with sheep receiving multiple injections triggered a reasonable 57% reduction in worm burden, which was verified by post-mortem analysis (Sexton *et al*, 1990).

In vitro studies show that employing of anti-GST antibodies from trial sheep does not affect inhibition of GST activity as well as reduction of fluke burden, suggesting that the observed protection is not because of inhibition of GST function (Morrison *et al*, 1996).

1.5.6. Drug resistance

In the mid-1990s, fluke resistance to triclabendazole was first documented on a sheep farm in Victoria, Australia (Overend & Bowen, 1995). Because triclabendazole binds to tubulin, mutations in tubulin were examined as a possible candidate mechanism (Robinson *et al*, 2002). However, in the resistant isolates Sligo and Oberon, no mutation linked to resistance was found (Ryan *et al*, 2008). Because triclabendazole is subject to bioactivation by oxidation to yield triclabendazole sulfoxide, resistance may arise by an alteration in the metabolic pathway (**Figure 5**) as : phase I (the activation phase, e.g., mainly cytochrome P450 enzymes), phase II (the conjugation phase, e.g., glutathione S-transferases), and phase III (the efflux phase, e.g., mostly ABC transporters). Conversion of triclabendazole to triclabendazole sulfoxide can be hampered by inhibitors of the cytochrome P450 (Devine *et al*, 2010, 2011, 2012) and by liver damage of the affected sheep (Overend & Bowen, 1995). However, it is not clear if cytochrome enzymes play a role in resistance. In contrast, flukes exposed to triclabendazole have increased activity of mu class GST (Fernández *et al*, 2014). Based on these results, the cDNA of GST-mu was amplified from a susceptible (Cullompton) strain and a resistant (Sligo) strain of *F. hepatica*. A change in the coding sequence resulting in the substitution of threonin¹⁴³ by serine was found in the Sligo strain (Fernández *et al*, 2015). However, the enzymatic activity of the protein was not determined. Hence it is not clear if this very conservative substitution resulted in a gain of a function capable of conferring resistance to triclabendazole sulfoxide. Finally, circumstantial evidence suggests that resistance arises from accelerated drug efflux, presumably via transmembrane transporters of the family of ABC transporters (Alvarez *et al*, 2005; Robinson *et al*, 2004). In fact, a gene encoding an ABC transporter of *F. hepatica* shows allelic variation: the allele encoding R¹¹⁴⁴ is threefold more prevalent in the triclabendazole-resistant fluke population than the

variant encoding serine at position 1144 (Wilkinson *et al*, 2012). However, the functional activity of the encoded protein was not determined. Thus, taken together, these studies suggest that several genes may be involved in triclabendazole resistance (Brennan *et al*, 2007), but the evidence is not convincing.

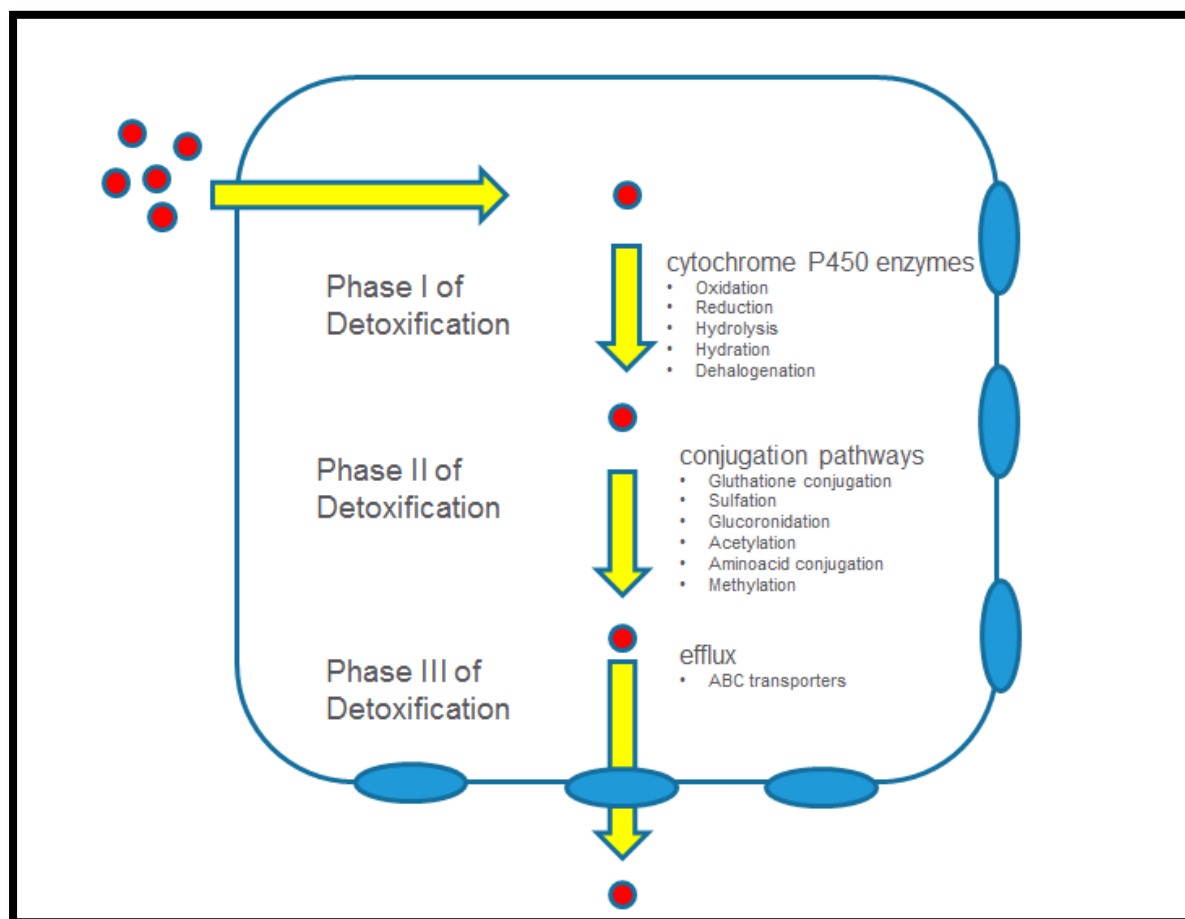


Figure 5. Illustration of the general detoxification pathway of metabolism/detoxification for a xenobiotic (shown as a red circle).

1.6. Bile acid metabolism

In the adult human liver, approximately 500 mg cholesterol is converted into bile acids each day. Both the steroid nucleus and the side chain of cholesterol are modified during bile acid synthesis, which results in a marked change in the physicochemical properties. The amphipathic property enables bile acids to emulsify lipids and enhance the absorption of lipids and fat-soluble vitamins in

the intestine. The detergent properties of bile acids are determined by the number and orientation of the hydroxyl groups and by the presence or absence of an amino acid moiety. The hydrophobicity increases as follows ursodeoxycholic acid (UDCA), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid (LCA) (Modica *et al*, 2010). Bile acid synthesis occurs in pericentral hepatocytes and involves enzymes located in the ER, mitochondria, cytosol, and peroxisomes (Russell, 2003, 2009). Bile acids are synthesized via two pathways, the classical or neutral and the alternative or acidic pathway. The classical pathway is the primary pathway of synthesis and accounts for more than 90% of the total bile acid synthesis in humans. In man, two bile acids are synthesized from cholesterol in the liver: CDCA (3 α , 7 α -dihydroxy) and CA (3 α , 7 α , 12 α -trihydroxy).

In the hepatocyte, bile acids are conjugated to glycine or taurine prior to secretion into the bile canaliculi (Hofmann & Mysels, 1992). In addition to amidation, bile acids may undergo sulphation, glucuronidation, and N-acetylamino-glucosidation. Conjugation increases the amphipathicity and enhances the solubility of the molecules, which makes them impermeable through cell membranes. Several bile acid transporter proteins ensure proper excretion and uptake. Bile acid transporters have different transport affinities for various bile acids species, but also for other endogenous and exogenous compounds such as toxins and drugs (Halilbasic *et al*, 2013). Efficient hepatic excretion, intestinal reabsorption, and hepatic uptake of bile acids restrict bile acids to hepatobiliary and intestinal compartments. Bile acid transporters and bile acid synthesis are subject to tight and coordinated regulation via nuclear receptors.

Bile acids are actively transported into bile canaliculi by the bile salt export pump (BSEP; encoded by the *ABCB11* gene). In the alkaline bile (pH 7.6-8.4), conjugated bile acids are present as ionic salts and therefore are often referred to as bile salts. Bile formation is an osmotic process and the most essential solutes driving bile formation are bile salts. To protect the canalicular membrane from bile acid-mediated solubilization, phospholipids are excreted into bile to form mixed micelles with bile acids (Elferink *et al*, 1997). Cholesterol excretion into bile is mediated by the ABCG5/ABCG8

heterodimer. Bile flows in bile canaliculi, which drain into bile ducts. The ducts join to form more extensive channels, the so-called biliary tree. A fraction of the bile acids are absorbed by cholangiocytes (the epithelial cells lining the biliary tract) and are recycled back to hepatocytes, the cholangiohepatic shunt (Alpini *et al*, 2005; Xia *et al*, 2006). The physiological relevance of this shunt is unclear.

1.7. Transporters

1.7.1. ABC transporters

ATP binding cassette (ABC) transporters comprise a large family of transmembrane proteins that bind their eponymous substrate ATP via two internally homologous nucleotide binding domains (NBD's) and use energy provided by ATP hydrolysis for vectorial pumping of a range of ligands. In many instances, ABC transporters function as an efflux pump with broad specificities for xenobiotics. ABC transporters utilize the hydrolysis of ATP to drive the transport of substrates against their concentration gradient. Generally, in prokaryotic cells, these transporters are importers, while in eukaryotic cells they function as exporters. The first eukaryotic transporter to be discovered, by its involvement in multidrug resistance (MDR), was P-glycoprotein (P-GP/MDR1/ ABCB1) (Chen *et al*, 1986; Gerlach *et al*, 1986; Gros *et al*, 1986; Riordan *et al*, 1985). The human genome encodes 48 ABC transporters. Mutations in these transporters can lead to a variety of genetic diseases, including bleeding disorders and liver and eye diseases (Martínez-Mir *et al*, 1998; Albrecht *et al*, 2005; Jacquemin, 2000).

ABC transporters confer resistance of tumor cells to cytotoxic chemotherapy and of bacteria and fungi to antibiotics (Alvarez *et al*, 2005; Robinson *et al*, 2004). Individual species differ regarding the number of ABC transporters depending on their ecological niche.

1.7.2. Solute carrier 6A6 (taurine transporter)

The human genome encodes more than 400 solute carriers (SLCs). The solute carrier 6 gene family encodes sodium/chloride (Na^+/Cl^-) dependent transporters. They represent one of the largest groups of the solute carrier superfamily. The SLC6 family is subdivided into four classes based on sequence similarities and substrate specificity (Fig. 6) (Kristensen *et al*, 2011) (i) the monoamine transporter branch with prominent representatives: dopamine (DAT), norepinephrine (NET) and serotonin (SERT) transporter; (ii&ii) amino acid transporters I including glycine transporters (GlyT) and II; and (iv) the gamma-aminobutyric acid (GABA) transporters (Bröer & Gether, 2012) (**Figure 6**).

The taurine transporter (TauT/SLC6A6) belongs to the family of GABA transporters (Bröer & Gether, 2012). It has a high-affinity for taurine (K_M 4-45 μM) and a low affinity for GABA (K_M 1.5 mM) (Kubo *et al*, 2016). The gene coding for the human taurine transporter is localized on chromosome 3 p24→p26. It encodes a 2864 bp long mRNA with a coding region of 1860 bp (Ramamoorthy *et al.*, 1994). The taurine transporter has the same structure as other Na^+/Cl^- coupled transporters of the SLC6 transporter family. It consists of 12 transmembrane spanning domains with intracellularly located N- and C-termini. Two potential N-glycosylation sites are present within the second extracellular loop. One potential phosphorylation site is present in the fourth intracellular loop, and the other four are located in the C-terminus (Uchida *et al*, 1992). Initially, the stoichiometry of symported co-substrate ions was reported as 3 Na^+ :1 Cl^- for taurine transport by rabbit jejunal brush border membranes (Miyamoto *et al.* 1989). However, a stoichiometry of 2 Na^+ : 1 Cl^- was determined for the cloned heterologously expressed human taurine transporter (Ramamoorthy *et al.*, 1994). The reason for this discrepancy is not apparent: it is unlikely that it can be attributed to species differences, and it is more likely that it reflects the inherent limitations of vesicular uptake (i.e., the presence of a mixture of right-side-out and inside-out vesicles, the poor control of the intravesicular ionic composition and the transmembrane potential due to the electrogenic nature of the substrate and co-substrate transport). In mammals, the taurine transporter is

found in all tissues, which have been examined suggesting that it is ubiquitously expressed albeit to different levels (Uchida *et al*, 1992; Smith *et al*, 1992; Ramamoorthy *et al*, 1994). The expression can be enhanced in the renal epithelial cell line MDCK by osmotic stress imposed by hypertonic solutions (Uchida *et al*, 1992). This regulation is recapitulated *in vivo* in the rat renal medulla, where expression of the taurine transporter increases upon salt loading of the animals (Bitoun *et al*, 2001). The promoter of the taurine transporter contains a tonicity-responsive element (TonE), which allows for the regulation of expression by TonEBP (TonE-binding protein) (Ito *et al*, 2004; Yang *et al*, 2015). TonEBP also allows for enhanced taurine transporter expression in response to inflammatory stimuli such as tumor necrosis factor- α (Johnson *et al*, 2017). Moreover, cellular taurine levels can apparently induce negative feedback on the expression of the taurine transporter: if HEPG2 cells (a cell line derived from a human hepatoma) are incubated in medium containing high taurine levels, the expression of the taurine transporter is markedly reduced (Satsu *et al*, 2003). *In vivo*, this effect is prominent in the kidney, in particular in the proximal straight part (S3 segment), where taurine depletion and taurine supplementation result in marked increases and decreases, respectively, of the mRNA encoding the taurine transporter (Matsell *et al*, 1997). The mechanism underlying this feedback regulation is not known. Finally, the promoter of the taurine transporter contains a p53 binding site, which mediates repression by activated p53 (Han *et al*, 2002). The physiological significance is not fully understood, but reduced expression of the taurine transporter in response to a proapoptotic stimulus is consistent with the cytoprotective role of intracellular taurine.

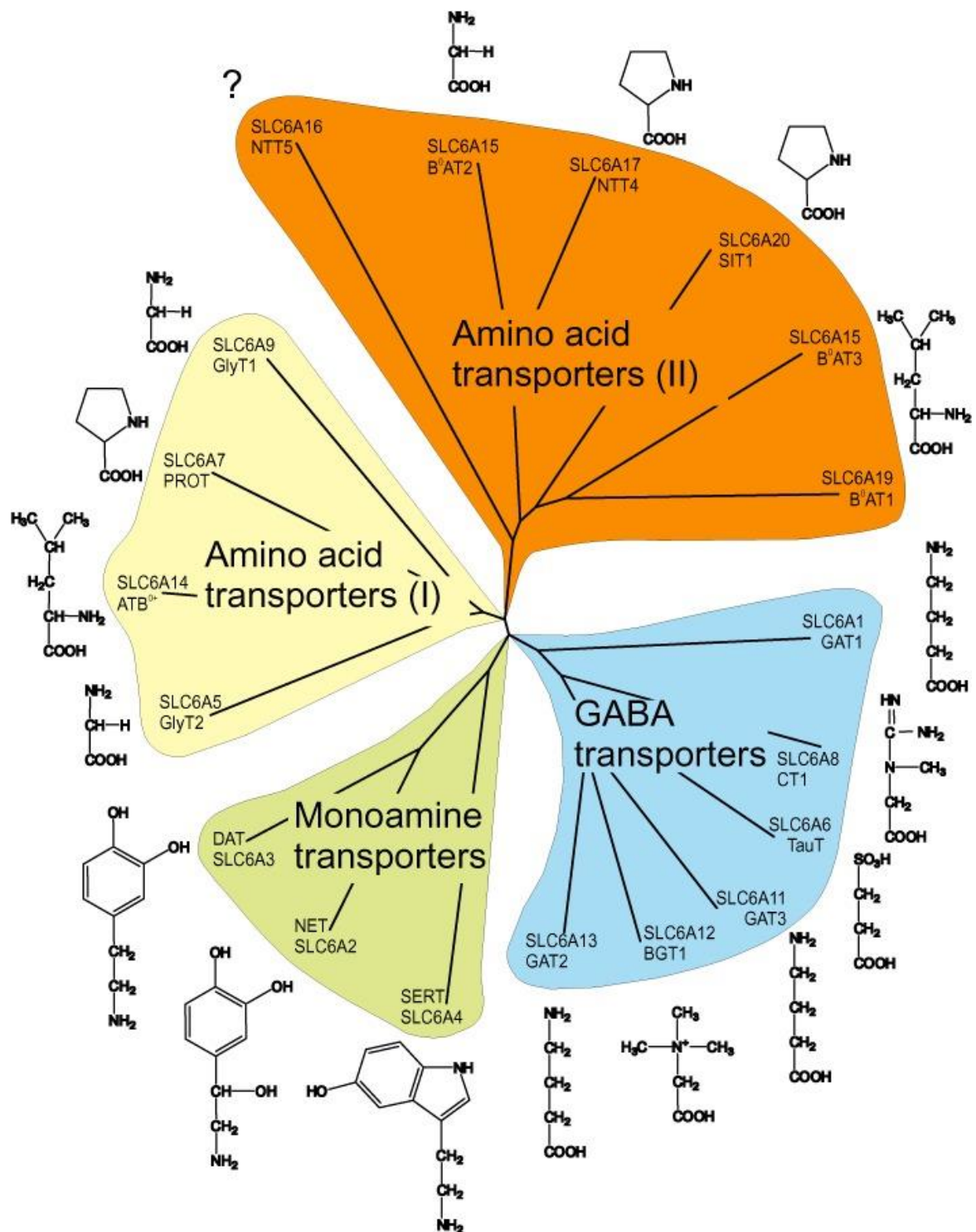


Figure 6. SLC6 transporter families and their sequence similarities. The figure is taken from Bröer & Gether, 2012.

1.8. Taurine

The term taurine was coined from the Latin/Greek term for bull (*Taurus/ταυρος*) by Friedrich Tiedemann and Leopold Gmelin (1827), who first extracted and purified the compound from ox bile. Taurine as a non-proteinogenic, sulfur-containing amino acid. Most animals are capable of synthesizing taurine from cysteine by the sequential action of cysteine dioxygenase, which yields cysteinesulfinate, and cysteine sulfinate decarboxylase, which converts cysteinesulfinate to hypotaurine; hypotaurine is oxidized enzymatically by hypotaurine dehydrogenase or nonenzymatically to taurine (Huxtable, 1992). The liver is the principal site of taurine synthesis (Ueki *et al*, 2011). However, the enzymes of the biosynthetic pathway are present in many organs; even in the absence of dietary taurine, the extrahepatic sites of taurine synthesis can compensate to maintain healthy levels of circulating taurine and of intrahepatic taurine, if the gene encoding the key enzyme cysteine dioxygenase is deleted explicitly in the liver (Ueki *et al*, 2012). This highlights the importance of the taurine transporter for replenishing intracellular taurine. Cats stand among mammals because they are deficient in taurine synthesis. Accordingly, they rely on a dietary taurine supply. Both, kittens and adult cats raised on a taurine-deficient diet develop a retinal degeneration within three months due to loss of photoreceptors (Hayes *et al*, 1975). This is also seen in mice, in which the gene encoding the taurine transporter has been deleted (Warskulat *et al*, 2007). In addition, the TauT^{-/-} mice display multiple abnormalities including reduced exercise capacity of the skeletal muscle, a slowly progressing liver fibrosis and multiple neurological deficits including loss of hearing and olfaction (Warskulat *et al*, 2007). Intracellular taurine levels are high: in many organs, in particular, the heart, brain, liver and skeletal muscle, taurine levels are in the range of 3 to 70 mmol/kg (Huxtable, 1992). Because of its zwitterionic and its highly hydrophilic nature, taurine can be readily accumulated within cells. In the cytoplasm, these properties make taurine ideally suited to act as an osmolyte, which protects cells against osmotic stress (Huxtable, 1992; Warskulat *et al*, 2007). In addition, taurine can act as an antioxidant by scavenging hypochlorite (Huxtable, 1992). Finally, taurine can also react with nucleobases to create 5-taurinomethyluridine and 5-

taurinomethyl-2-thiouridine, which are found in mitochondrial tRNA (Suzuki *et al*, 2002); the modification promotes codon/anticodon interactions in mitochondrial ribosomes; in the absence of these taurine-dependent modifications the codons for leucine (UUG), lysine (AAG), glutamate (GAG) and glutamine (CAG) are translated inefficiently, which gives rise to mitochondrial diseases (Schaffer *et al*, 2014). Reduced taurine-dependent modifications are seen in point mutations of mitochondrial DNA encoding tRNAs, which are referred to by the acronyms MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) and MERRF (myoclonic epilepsy and ragged-red fiber syndrome). These syndrome phenocopy taurine deficiency, which indicates that the modification of mitochondrial tRNA by taurine is of major importance in the brain, retina, skeletal muscle, and so forth. (Schaffer *et al*, 2014).

1.9. Aims of this thesis

Taurine is present throughout the animal kingdom and can be found at high levels within various non-chordate species including insects and molluscs (Huxtable 1992). Taurine is also present in *Fasciolidae* (Tandon, 1968).

The working hypothesis underlying the present thesis posits that *F. hepatica* expresses a taurine transporter as a defense mechanism, which allows adult flukes to survive in the hostile environment of the bile ducts. While adult flukes feed mainly on blood via their oral sucker, their tegument is also known to allow for solute uptake (Dalton *et al*. 2004). For an active defense, taurine transport must also be present in the tegumental cells. If this conjecture is correct, the taurine transporter can be predicted to have biochemical properties indicative of adaptation to the milieu of the host bile. Similar, the working hypothesis predicts that taurine ought to enhance survival of flukes challenged by bile acids. Conversely, inhibition of taurine uptake by *F. hepatica* ought to blunt the protective action of taurine. Thus, the present thesis aimed to identify the taurine transporter of *F. hepatica* and to verify the predictions of the working hypothesis.

2. Results

Identification and Characterization of the *Fasciola hepatica* Sodium- and Chloride-Dependent Taurine Transporter

Bulut Hamali, Sandra Pichler, Elisabeth Wischnitzki, Klaus Schicker, Melanie Burger, Marion Holy, Kathrin Jaentsch, Martina Molin, Eva Maria Sehr, Oliver Kudlacek, and Michael Freissmuth.
PLOS Neglected Tropical Disease. (submitted)



Identification and Characterization of the *Fasciola hepatica* Sodium- and Chloride-Dependent Taurine Transporter

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Abstract

The parasitic liver fluke *Fasciola hepatica* infests mainly ruminants, but it can also cause fascioliasis in people, who ingest the metacercariae encysted on plants. The drug of choice to treat fascioliasis is triclabendazole (TBZ), which has been on the market for several decades. This is also true for the other available drugs. Accordingly, drug-resistant flukes have been emerging

at an increasing rate making it desirable to identify alternative drug targets. Here, we focused on the fact that adult *F. hepatica* persists in the hostile environment of the bile ducts of infected organisms. A common way to render bile acids less toxic is to conjugate them to taurine (2-aminoethanesulfonic acid). We cloned a transporter from the solute carrier-6 (SLC6) family, which was most closely related to the GABA-transporter-2 of other organisms. When heterologously expressed, this *F. hepatica* transporter supported the high-affinity cellular uptake of taurine ($K_M=12.0 \pm 0.5 \mu\text{M}$) but not of GABA. Substrate uptake was dependent on Na^+ and Cl^- (calculated stoichiometry 2:1). Consistent with the low chloride concentration in mammalian bile, the *F. hepatica* transporter had a higher apparent affinity for Cl^- ($\text{EC}_{50} = 14 \pm 3 \text{ mM}$) than the human taurine transporter ($\text{EC}_{50} = 55 \pm 7 \text{ mM}$). We incubated flukes with unconjugated bile acids in the presence and absence of taurine: taurine promoted survival of flukes; the taurine transporter inhibitor guanidinoethanesulfonic acid abolished this protective effect of taurine. Based on these observations, we conclude that the taurine transporter is critical for the survival of liver flukes in the bile. Thus, the taurine transporter represents a candidate drug target.

Author Summary (150 -200 words)

The liver fluke *Fasciola hepatica* imposes an economic burden by infesting domestic ruminants. In addition, the WHO classified human fascioliasis as a disease of vital global public health importance, because several million people worldwide are infested by this trematode. Because it is currently not possible to vaccinate against this parasite, anthelmintic drugs are the treatment of choice for both, animals and people. During the last decades, the proportion of flukes resistant to drugs has steadily increased. *F. hepatica* persists in the hostile environment of the host bile duct. Accordingly, the fluke must be endowed with defense mechanisms, which protect it against the toxic actions of bile acids.

This working hypothesis was explored by cloning a candidate taurine transporter of *F. hepatica*. The experiments verified that the transporter-mediated the sodium- and chloride-dependent uptake of taurine. It requires only low concentrations of chloride, which indicates adaptation to the ionic composition of the bile, and it is protective: inhibition of the transporter renders flukes susceptible to killing by bile acids. By showing that the taurine transporter represents an Achilles heel of *F. hepatica*, these observations point to a new anthelmintic therapeutic strategy.

Introduction

Liver flukes of the genus *Fasciola* are parasitic trematodes, which infest mammals all over the world. The two most prominent representatives of the genus *Fasciola* are *Fasciola gigantica* and *Fasciola hepatica*, which predominates in the temperate zones and tropical Africa and Asia, respectively [1]. The resulting fascioliasis imposes a substantial economic burden because of the decrease in milk production, weight gain and wool yield and due to sudden deaths of livestock animals [2–5]. People are infested accidentally by ingestion of metacercariae encysted on plants or ingestion of water containing metacercariae. This not only can give rise to regional pockets of endemic infections, e.g., on the Bolivian Altiplano [6] but is also relevant to human health worldwide: conservative estimates indicate that more than 2.5 million people are infested and suffer from various forms of fascioliasis [7].

A vaccine against *F. hepatica* is desirable, but there are many obstacles, which impede the development of an effective active immunization [8]. Accordingly, fascioliasis is treated by anthelmintic chemotherapy. Triclabendazole has been the drug of choice for more than 35 years: triclabendazole is highly active against both adult and juvenile flukes and is well tolerated by the mammalian hosts. Predictably, flukes resistant to triclabendazole have emerged [9].

Resistance is also seen with other anthelmintic compounds, which kill *F. hepatica*, e.g. albendazole, clorsulon, closantel, oxiclozanide and nitroxynil [9–11]. Most mammals do not mount an effective immune response to liver flukes [8,12]. Hence adult *F. hepatica* can survive for many years within the biliary tract: adult worms were retrieved after 11 years from an experimentally infected sheep [13]. A case report from an elderly patient suggests that *F. hepatica* can also reach this age in people [14]. Motile flukes have been visualized by radiological imaging techniques in the gall bladder and in the common bile duct of patients [15]. In fact, whole bile and some bile acids such as glycine-conjugated cholic acid and dehydrocholic acid stimulate the mobility of juvenile flukes [16,17]. This indicates that some bile acids provide a chemokinetic or chemotactic signal. However, bile acids, in particular, deoxycholic acid, are also toxic to flukes [17]. Thus, while juvenile and adult *F. hepatica* are attracted by bile constituents, protective mechanisms must have evolved, which allow them to survive the toxic effects of the bile, e.g. by conjugating taurine to bile acids. Here we surmised that flukes rely on taurine transport to cope with bile acids. We cloned a candidate taurine transporter, confirmed its biochemical activity and verified that inhibition of taurine transport rendered adult flukes susceptible to killing by bile acids.

Results

Primary structure of the *F. hepatica* taurine transporter (FhTauT)

Transcripts encoding for the orthologue of the human taurine transporter SLC6A6 were identified from a sequence database including mRNA data of adult *F. hepatica* [18]. A fragment of about 0.8 kB was identified via the sequence analysis and thereafter extended by amplifying the cDNA ends. An in-frame stop codon is present 5 triplets upstream of the first ATG (Fig. 1), which indicates that the translation start site has been correctly identified. In addition, the sequence flanking the first ATG conforms to a canonical Kozak sequence with guanine bases at positions -6, -3 and +4. The 3' end of the amplified fragment also comprised the polyadenylation signal

(starting with A2746, Fig. 1A) and the initial portion of the poly-A tail indicating that the amplified fragment covered the entire mRNA. The open reading frame of 1944 bp encodes a protein of 647 amino acids (Fig. 1A) corresponding to a relative molecular mass of 71,939 Da. The sequence was deposited at GenBank with the accession number: MG674191 as *F. hepatica* taurine transporter (FhTauT). The exon-intron boundaries were identified by comparing the obtained cDNA sequence with the genomic sequence deposited in the database wormbase (<http://parasite.wormbase.org/>) [19,20]. This analysis led to the prediction that the FhTauT was encoded by 14 exons (Fig. 1B).





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(<http://parasite.wormbase.org/>) using the Splign web tool (<https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi>). The figure was generated by ExonIntron Graphic Maker (<http://www.wormweb.org/exonintron>). The scale bar represents 10 kb.

We aligned the amino acid sequence of the putative taurine transporter of *F. hepatica* to that of three sequences of human SLC6 family members - i.e. of the serotonin transporter (SERT/SLC6A4), the GABA transporter-2 (GAT2/SLC6A13), the taurine transporter (TauT/SLC6A6) - and of the putative GABA-transporter-2 of the trematode *Clonorchis sinensis*, another liver fluke. It is evident from Fig. 2 that there is extensive sequence conservation within the hydrophobic core, which is comprised of the twelve transmembrane (TM) segments. The structure of several SLC6 transporters is understood in atomic detail. In the crystal structure of SERT [21], the Na⁺ binding site is comprised of A⁹⁶ and N¹⁰¹ in TM1, S³³⁶ in TM6 and N³⁶⁸ in TM7 (marked by red arrow heads in Fig. 2): these residues (i.e. N⁷³, S³⁰³ and N³³⁵) are conserved in the *F. hepatica* taurine transporter with the exception of A⁹⁶, which is replaced by S⁶⁸. Similarly, the residues forming the binding site of the second sodium ion in human SERT (G⁹⁴ and V⁹⁷ in TM1, L⁴³⁴, D⁴³⁷ and S⁴³⁸ in TM8 marked by blue arrow heads in Fig. 2), are identical in the *F. hepatica* taurine transporter (G⁶⁶, V⁶⁹, L⁴⁰¹, L⁴³⁴, S⁴³⁵). In SERT, the chloride ion is coordinated by Y¹²¹ of TM2, Q³³² and S³³⁶ of TM6 and S³⁷² of TM7 (marked by green arrow heads in Fig. 2). These residues are also invariant (Y⁹³, Q²⁹⁹, Q³⁰³, S³³⁹). SLC6 transporters have a long second extracellular loop (EL2), which is stabilized by a disulfide bond. The candidate cysteine residues (C¹⁷¹ and C¹⁸¹ marked by magenta arrow heds in Fig. 2) are also present in the *F. hepatica* taurine transporter. SLC6 transporters have a variable number of N-linked glycosylation sites; SERT has a single site (Fig. 2), but the dopamine transporter DAT (SLC6A3) has three [22,23]. We identified two asparagine residues which conform to the NXS/T-glycosylation motif in the *F. hepatica* taurine transporter (N¹⁸³ and N¹⁸⁹, marked by brown arrow heads in Fig. 2). Finally, with the notable

exception of the neutral amino acid transporters B⁰AT3/SLC6A18 and B⁰AT1/SLC6A19 [24], SLC6 transporters harbor a SEC24-binding site in their C-terminus: this RI/RL-motif [25–27] is also present in the *F. hepatica* taurine transporter (R579/I580, black arrow heads in Fig. 2). The +2 residue (T⁵⁸²) is hydrophilic, which predicts that the *F. hepatica* transporter recruits SEC24C for ER-export [27].



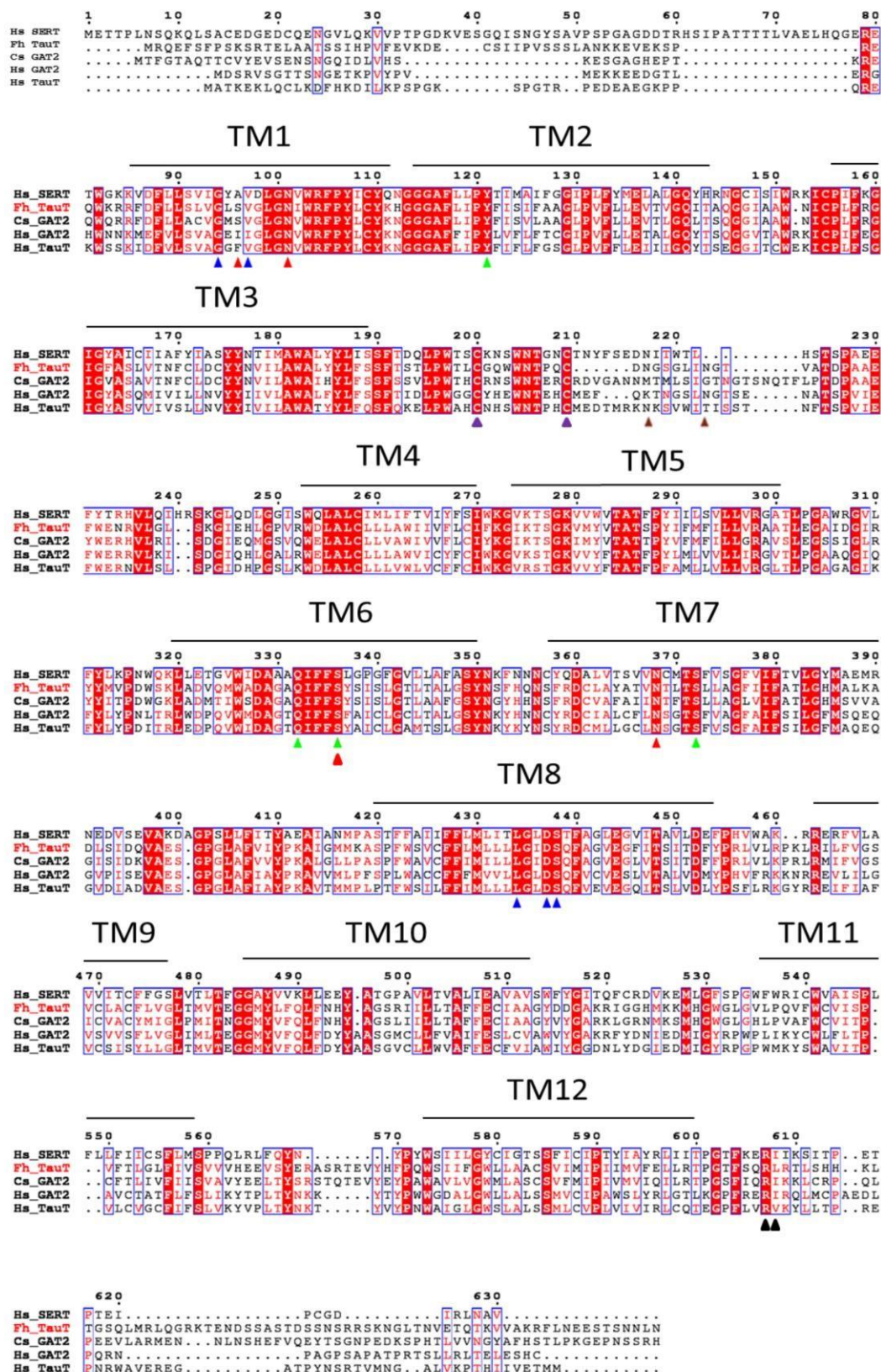


Fig. 2 Amino acid alignment of the *F. hepatica* taurine transporter (Fh TauT) with the human serotonin transporter (Hs SERT), the GABA-transporter-2 of *Clonorchis sinensis* (Cs GAT2), the human GABA-transporter-2 (Hs GAT2) and the human taurine transporter (Hs TauT). Amino acids are represented in their single-letter code. Invariant sequences are denoted by white letters boxed in red,

sequences with conservative substitutions are denoted by red letters. The red and blue arrow heads mark amino acids contributing to the first and second Na⁺ binding sites, respectively, in the crystal structure of SERT. The green arrow heads highlight residues involved in the coordination of Cl⁻ in SERT. The cysteine residues in the second extracellular loop, which form a putative disulfide bridges, are indicated by magenta arrow heads. The brown arrow heads indicate candidate sites for N-linked glycosylation predicted by NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>).

Phylogenetic analysis of the putative *F. hepatica* transporter

We used the basic local alignment tool (BLAST) to identify the closest relatives of the putative *F. hepatica* taurine transporter. As expected, this search retrieved SLC6 transporters from other digenean trematodes - i.e., *Clonorchis sinensis*, *Opisthorchis viverrini*, *Schistosoma mansoni*, *Schistosoma haematobium* - and taurine or GABA transporters from other species (e.g., the deep-sea mollusc *Bathymodiolus septemdierum*, *Drosophila melanogaster* and *Homo sapiens*). We selected these representative sequences to construct a phylogenetic tree (Fig. 3) by the neighbor-joining method [28]. The GABA transporter 2 from *Clonorchis sinensis* and the *F. hepatica* taurine transporter showed the highest pairwise identity of 65 %. The evolutionary relation of digenean trematodes has been clarified based on the comparison of 12 proteins encoded by their mitochondrial genomes [29]: this shows that Opisthorchiidae and Fasciolidae (and Paragonimidae) are closely related, while flukes of the Schistosomatidae family are assigned to a separate branch. The evolutionary tree depicted in Fig. 3 only recapitulated this relation in part, because the (hypothetical) SLC6 transporter from *Opisthorchus viverrini* was assigned to the same branch as the *Schistosoma* transporters for GABA and serotonin. However, it is worth pointing out that the biochemical activity of the majority of these transporters has only been inferred: in fact, of the non-human transporter shown in Fig. 3, it is only clear that the taurine transporter of *Bathymodiolus septemdierum* mediates the uptake of its eponymous substrate [30]. It is therefore questionable that the assigned

names are correct: it is, for instance, difficult to understand, why the serotonin transporter of *Schistosoma mansoni* should be more closely related to the *Schistosoma mansoni* GABA-transporter-2 than to the human serotonin transporter (Fig. 3).

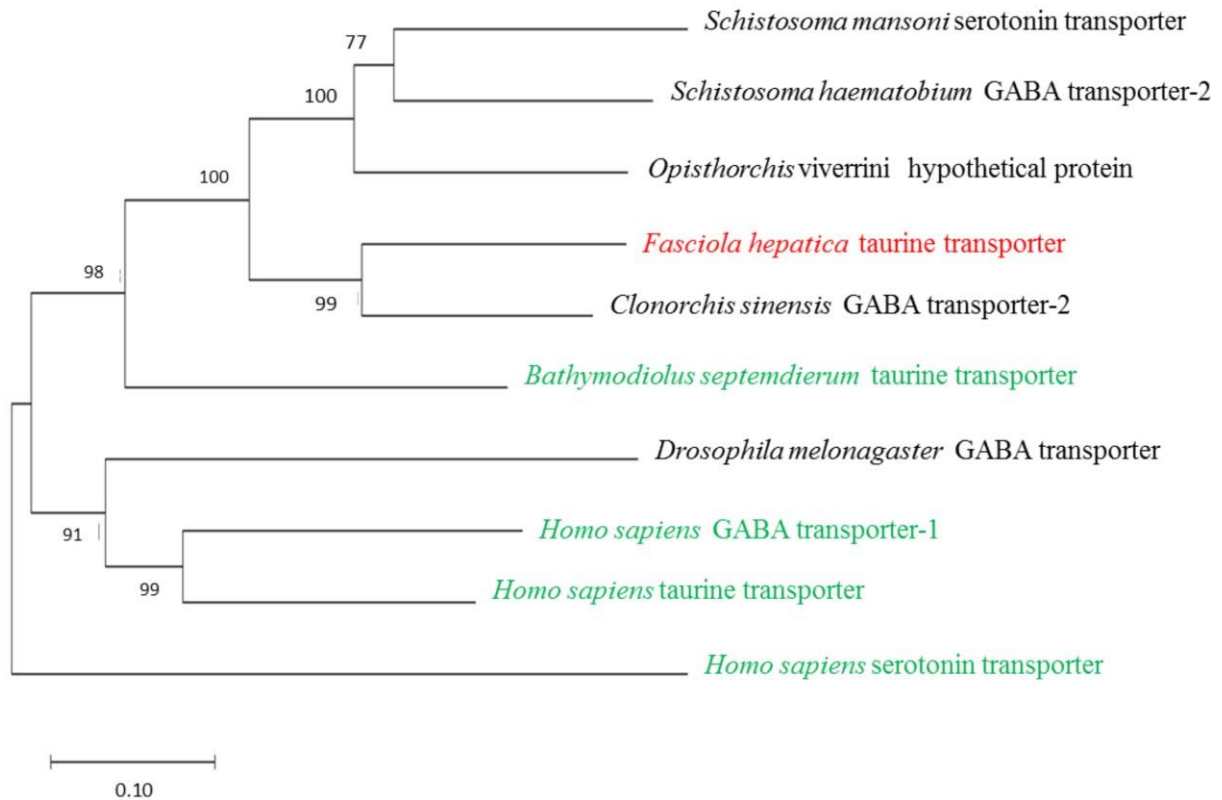


Fig. 3. Phylogenetic relation of the *Fasciola hepatica* taurine transporter against other organism transporters. The neighbor-joining method was used to construct the phylogenetic tree by comparing amino acid sequences of transporters (for accession numbers of sequences see materials and methods) with MEGA7 [31]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates, Poisson correction) are shown next to the branches. The scale bar reflects the likelihood that a change in amino acid occurred on any given branch. The transporters written in green indicate those SLC6 family members, where the substrate uptake has been verified in biochemical experiments and/or by electrophysiological recordings. For transporters written in black the functional assignment is based on sequence homology.

Heterologous expression of the *F. hepatica* taurine transporter in HEK293 cells

The sequence comparison in Fig. 2 and the evolutionary tree in Fig. 3 highlight the close relation between taurine and GABA transporters. We verified the substrate specificity of the putative *F. hepatica* transporter by heterologous expression in

HEK293 cells. The human taurine transporter was used as a reference. SLC6 transporters can be tagged with fluorescent proteins on their N-terminus without affecting their activity [32] and their trafficking through the secretory pathway [33–35]. Accordingly, we introduced the cDNA encoding the *F. hepatica* and the human taurine transporter into mammalian expression vectors, which fused the coding sequence of CFP and YFP, respectively, in frame to the N-terminus of the transporters. This allowed for visualizing the cellular distribution of the transporters by confocal microscopy after heterologous expression in HEK293 cells: the plasma membrane was delineated by staining with trypan blue. A larger fraction of the *F. hepatica* taurine transporter (Fig. 4A) than of the human transporter (Fig. 4B) seems to be retained within the cell. However, both transporters also reached the cell surface. Accordingly, cells expressing the *F. hepatica* and the human taurine transporter and untransfected control cells were incubated in the presence of [³H]taurine and of [³H]GABA to determine the substrate specificity. We failed to detect any uptake of [³H]GABA (data not shown). In contrast, cells expressing the *F. hepatica* accumulated substantially (i.e., about 10-fold) higher amounts of [³H]taurine (closed circles in Fig. 4C) than untransfected control cells (open triangles in Fig. 4C). Transport by both, the *F. hepatica* (Fig. 4C) and the human transporter (Fig. 4D) was adequately described by a rectangular hyperbola. The K_M of the human transporter was $41.0 \pm 10.4 \mu\text{M}$, which is excellent agreement with that originally reported in a similar uptake assay ($43 \pm 6 \mu\text{M}$) [36]. The apparent affinity of the *F. hepatica* transporter for taurine ($K_M = 12.0 \pm 0.5 \mu\text{M}$) was higher than that of the human transporter. In contrast, the maximum velocity of uptake was lower in cells expressing the *F. hepatica* taurine transporter ($178 \pm 2 \text{ pmol} \cdot 10^{-6} \text{ cells} \cdot \text{min}^{-1}$) than in cells expressing the human taurine transporter ($971.0 \pm 60.3 \text{ pmol} \cdot 10^{-6} \text{ cells} \cdot \text{min}^{-1}$, Fig. 4D). This can be accounted

for - at least in part - by the lower surface expression of the *F. hepatica* taurine transporter (Fig. 4A).

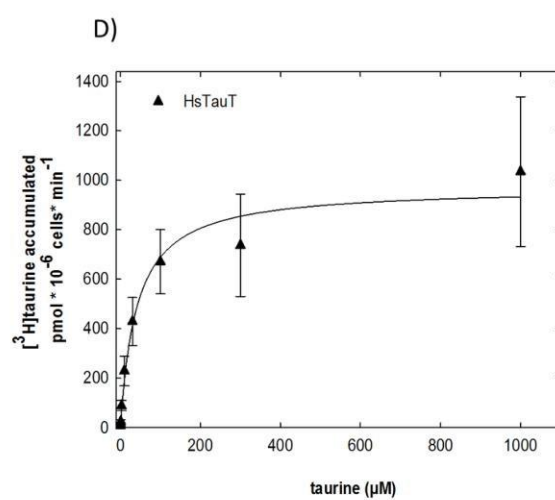
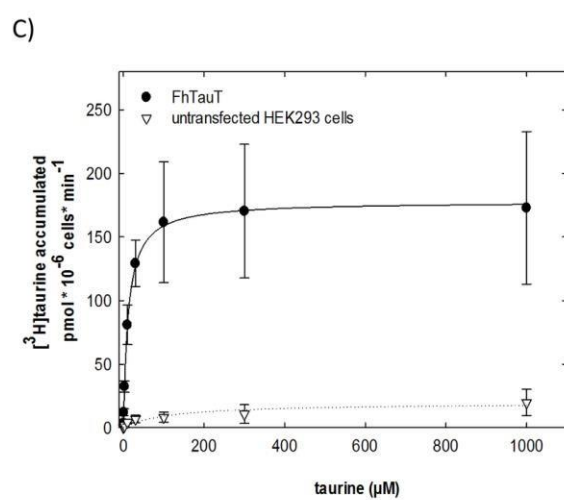
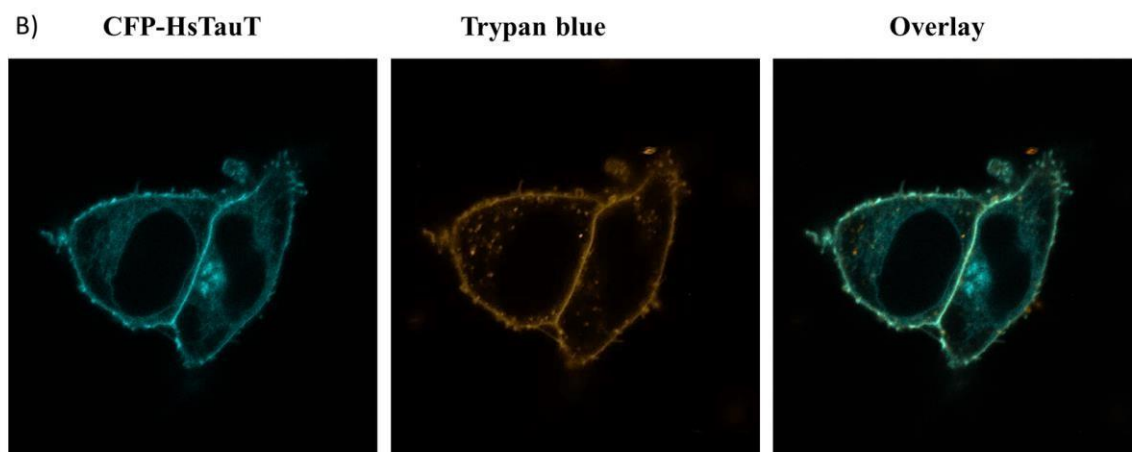
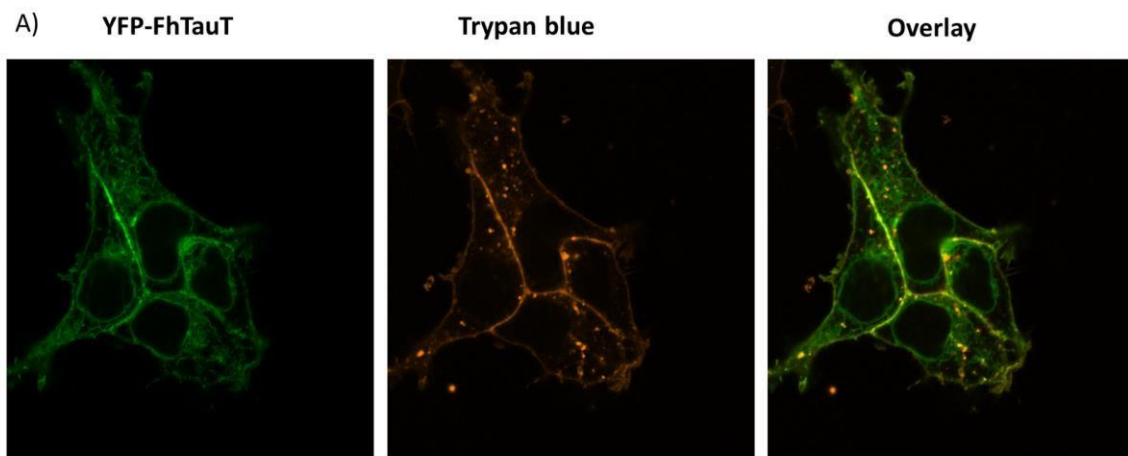
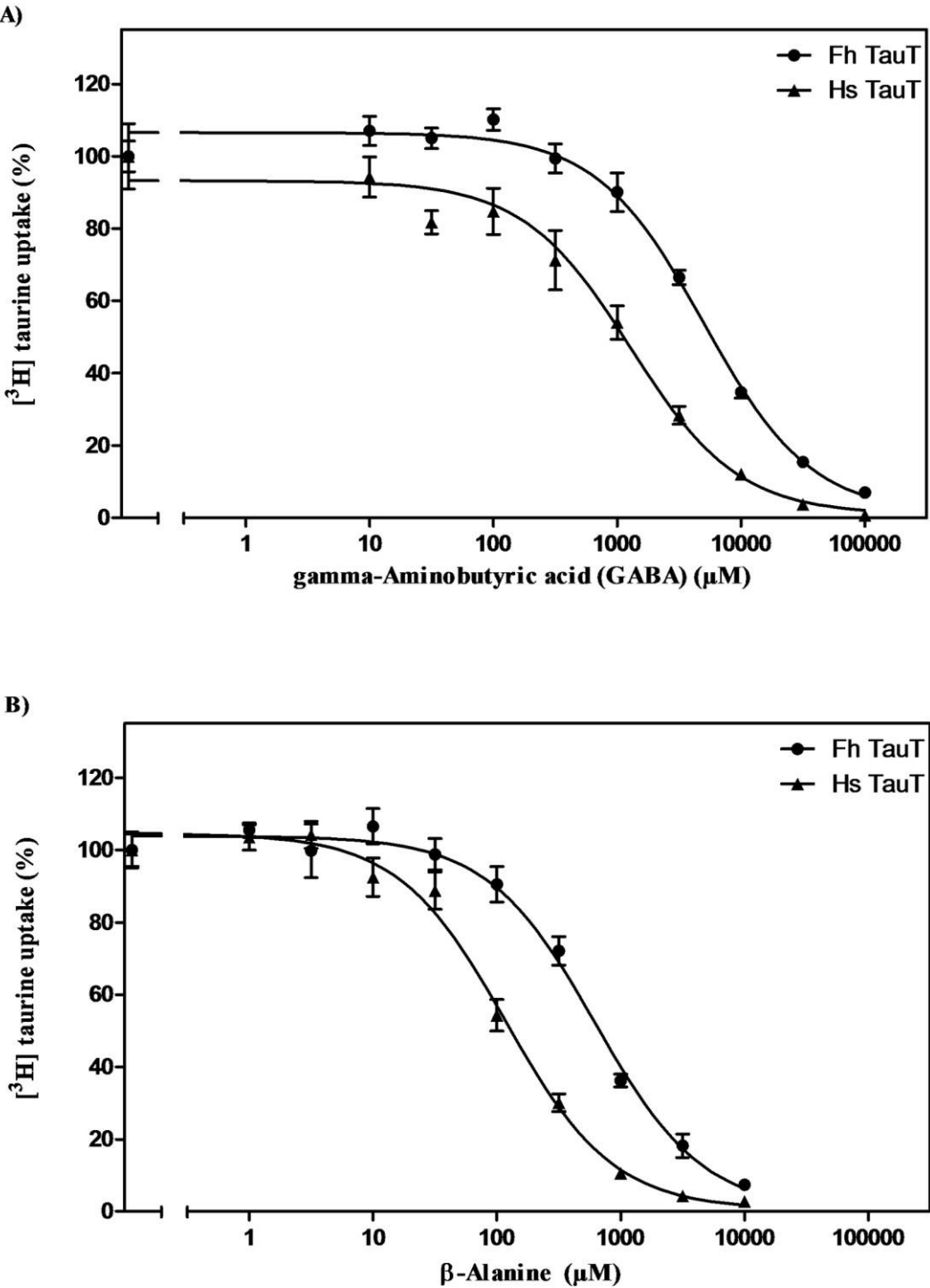


Fig. 4. Cellular localization of (A, B) and substrate uptake (C, D) by the *F. hepatica* (A , C) and the human taurine transporter (B , D) after heterologous expression in HEK293 cells. HEK293 cells were stably transfected with plasmids encoding fluorescently tagged versions of the *F. hepatica* (YFP-FhTauT, panel A) and the human (CFP-HsTauT, panel B). The cellular distribution of the tagged transporters was visualized by confocal microscopy (left-hand images). The cell surface was delineated by staining with trypan blue (images in the middle). The captured confocal images were overlaid (right-hand images). *C & D*: HEK 293 cells (1×10^5 cells/well) expressing the YFP-tagged *F. hepatica* taurine transporter (circles in C) and the YFP-tagged human taurine transporter (circles in D) untransfected HEK 293 cells (open triangles in C) were incubated for 10 min in the presence of the indicated concentration of [^{3} H]taurine. The accumulated radioactivity was determined as described under *Materials and Methods*. Data are means $\pm$ S.D. of at least three independent experiments, which were carried out in duplicate. The solid lines were drawn by fitting the data to the equation of a rectangular hyperbola.

We also examined the ability of unlabelled GABA to inhibit uptake of [^{3} H]taurine to estimate the affinity of the *F. hepatica* transporter for GABA: High concentrations of GABA were required to suppress taurine uptake by the *F. hepatica* transporter (Fig. 5A). From the monophasic inhibition curves, we calculated IC_{50} -values of 5.6 ± 0.9 mM. As a reference, we assessed the affinity of the human taurine transporter for GABA in parallel (triangles in Fig. 5A): GABA was more potent in inhibiting the human than the fluke transporter; the estimated IC_{50} of GABA (1.6 ± 0.5 mM) is in agreement with the reported K_M of 1.46 mM [37]. β -Alanine was originally proposed as the alternative substrate of the taurine transporter [38]. Accordingly, we also compared the apparent affinity of the human and the *F. hepatica* transporter for β -alanine (Fig. 5B): β -alanine was more potent in inhibiting [^{3} H]taurine uptake by the human transporter ($IC_{50} = 132 \pm 54$ μ M) than by the fluke transporter ($IC_{50} = 713 \pm 260$ μ M). The affinity estimate for the human transporter was in

line with the original observation that 100 μM β -alanine inhibited uptake by about 50% [36].



265 Fig. 5. GABA- (A) and β -alanine-induced (B) inhibition of [^3H]taurine uptake by the *F.*

***hepatica* and the human taurine transporter after heterologous expression in HEK293 cells.** Stably transfected HEK 293 cells (1×10^5 cells/well) expressing the YFP-tagged *F. hepatica* taurine transporter (circles, YFP-FhTauT) and the YFP-tagged human taurine transporter (triangles, CFP-HsTauT) were incubated for 10 min in the presence of $0.1 \mu\text{M}$ [^3H]taurine and the indicated concentration of GABA (A) and β -alanine (B). The accumulated radioactivity was determined as described under Materials and Methods. Uptake observed in the absence of any inhibitor was set 100% to normalize for interassay variability. These 100% control values were 0.89 ± 0.05 and $1.102 \pm 0.142 \text{ pmol} \cdot \text{min}^{-1} \cdot 10^{-6}$ cells for the *F. hepatica* and the human transporter, respectively. Data are means $\pm$ S.D. from three independent experiments. The solid lines were drawn by fitting the data to the equation for a monophasic inhibition curve.

Substrate transport by SLC6 family members requires the co-transport of two or three Na^+ ions. These provide the driving force for intracellular accumulation of the substrate. In addition, most SLC6 transporters are dependent on chloride, although the chloride gradient is immaterial, because the transporter completes the transport cycle in a chloride-bound return step [39]. We compared the ionic requirement of the *F. hepatica* (Fig. 6A&C) and the human transporter (Fig. 6B&D) by isoosmotic replacement of sodium with choline (Fig. 6A&B) and of chloride with acetate (Fig. 6C&D). Both transporters required similar amounts of Na^+ for half-maximum stimulation of transport ($\text{EC}_{50} = 51.4 \pm 6.0 \text{ mM}$ and $56.5 \pm 7.1 \text{ mM}$ for the *F. hepatica* and the human transporter, respectively). Similarly, the sodium saturation curves were sigmoidal with Hill coefficients of 1.6 ± 0.1 and 2.3 ± 0.1 for the *F. hepatica* and the human transporter, respectively. In contrast, the chloride saturation curves were hyperbolic (Fig. 6C&D). It is also evident from the comparison of Fig. 6C and Fig. 6D that substrate uptake by the *F. hepatica* transporter required substantially lower concentrations of chloride ($\text{EC}_{50} = 13.9 \pm 3.1 \text{ mM}$) than the human transporter ($54.8 \pm 6.9 \text{ mM}$).

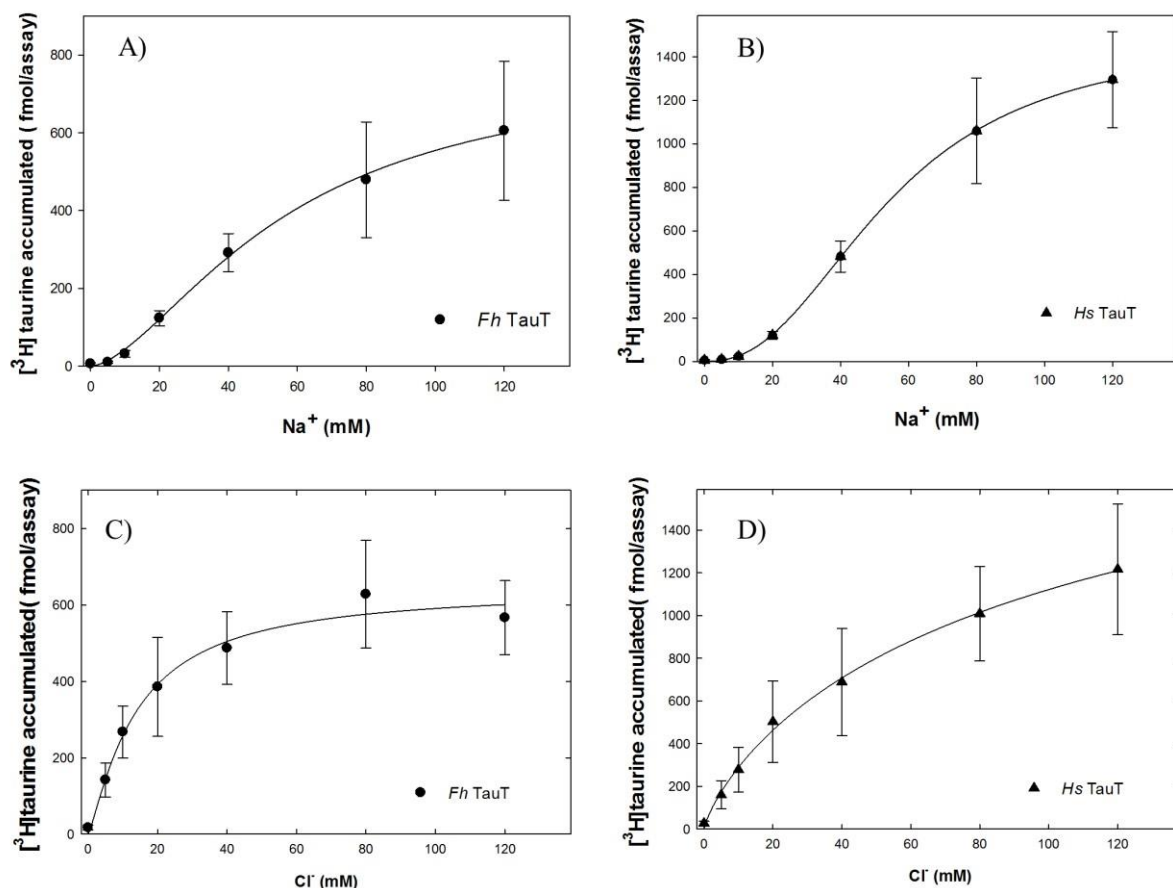


Fig. 6. Sodium- (A, B) and chloride-dependence (C, D) of [³H]taurine uptake by the *F. hepatica* and the human taurine transporter. HEK293 cells (1×10^5 cells/well) stably expressing the *F. hepatica* (A, B) or the human taurine transporter (C, D) were incubated for 10 min in the presence of 0.1 μ M [³H]taurine and of the indicated concentrations of sodium (A, B) and chloride (C, D). The ionic strength was kept constant by replacing sodium with choline (A, B) or by substituting chloride with acetate (C, D). Data represent means $\pm$ S.D. from three independent experiments carried out in duplicate. The solid lines were drawn by fitting the data to the Hill equation.

The number of compounds, which act as specific inhibitors of taurine uptake, is very limited. Because of the close relation between GABA- and taurine transporters, we examined several compounds, which act as inhibitors of GABA uptake or of GABA degradation (e.g., nipecotic acid, tiagabine, vigabatrin), but these failed to inhibit the *F. hepatica* transporter. In fact, the only compound other than GABA and β -alanine (cf. Fig. 5) was guanidinoethyl sulfonate (taurocyamine or GES). This compound was classified as a competitive inhibitor of mammalian taurine transporters [40]. We verified the mode of inhibition by determining uptake of [³H]taurine by cells expressing the *F. hepatica* (Fig. 7A) and the human transporter (Fig. 7C) in the presence of increasing concentrations of unlabeled taurine and of GES: in cells expressing the human

transporter, the action of GES was consistent with competitive inhibition, because the IC_{50} of unlabeled taurine was progressively shifted to the right (Fig. 7C; IC_{50} = $41.2 \pm 8.2 \mu\text{M}$, $52.1 \pm 9.5 \mu\text{M}$ and $98.9 \pm 20.1 \mu\text{M}$ in the absence and presence of 0.1 and 0.3 mM GES, respectively). Accordingly, when the data were replotted in a Dixon plot, the resulting lines were parallel indicating that binding of taurine and GES was mutually exclusive (Fig. 7D). The affinity of the *F. hepatica* transporter for GES was about ten-fold lower than that of the human transporter; this can be seen by comparing the uptake in the absence of unlabeled taurine in Fig. 7A and Fig. 7C, which was about 75% and 40% in the presence of 1 mM and 3 mM GES, respectively, for the *F. hepatica* transporter (Fig. 7A). Equivalent residual transport were seen at 0.1 and 0.3 mM GES with the human transporter (Fig. 7C). In addition, GES was a noncompetitive inhibitor of taurine uptake by the *F. hepatica* transporter: the IC_{50} of unlabeled taurine were not shifted to the in the presence of GES (Fig. 7A; IC_{50} = $11.7 \pm 4.1 \mu\text{M}$, $18.7 \pm 6.7 \mu\text{M}$, and $16.0 \pm 4.2 \mu\text{M}$ in the absence and presence of 1 and 3 mM GES, respectively. Transformation of these data into a Dixon plot produced a family of intersecting lines. This indicates a non-competitive inhibition, where taurine and GES can be bound simultaneously.

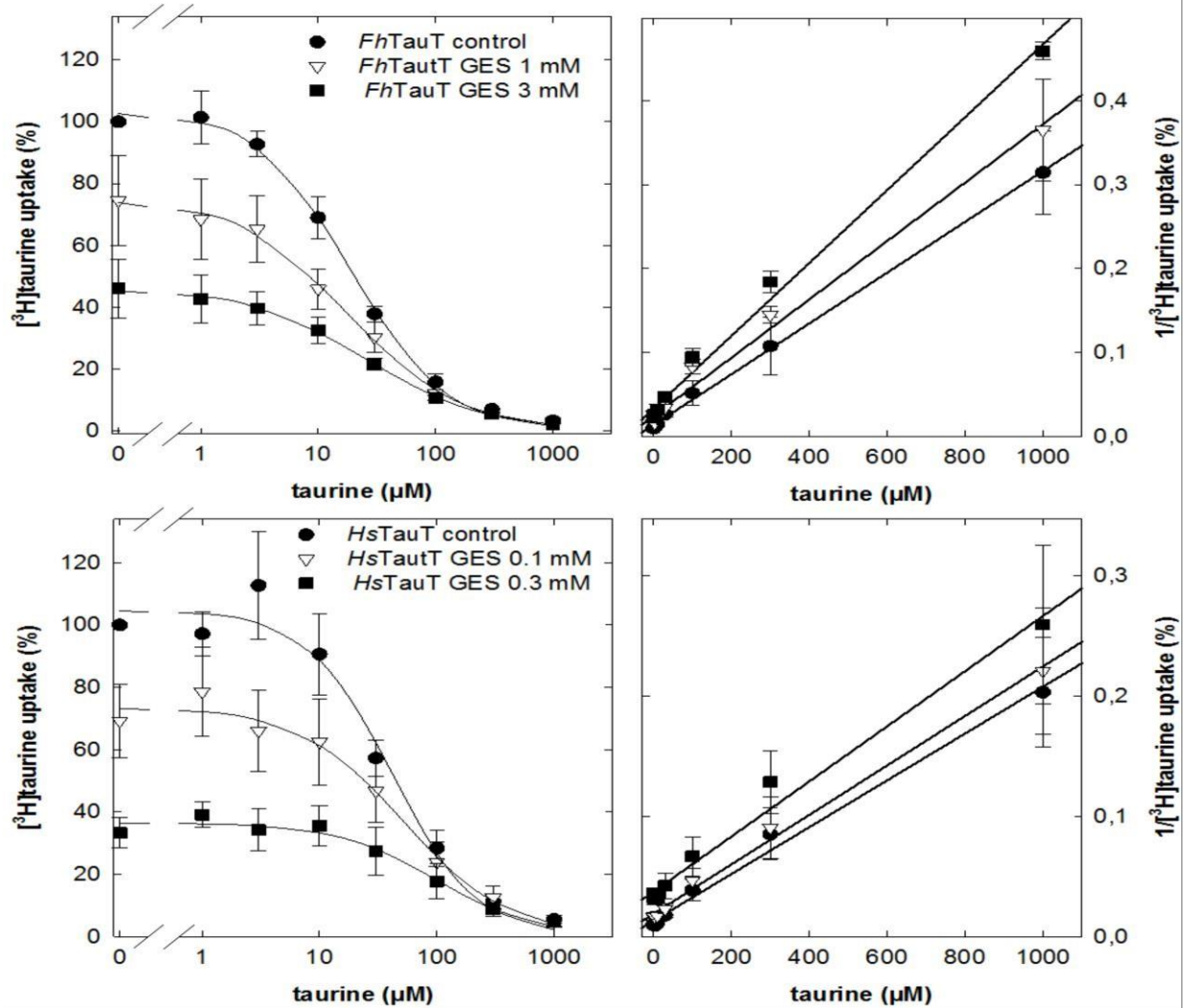


Fig. 7. Inhibition of $[^3\text{H}]$ taurine uptake by unlabeled taurine in the absence and presence of GES. HEK293 cells (1×10^5 cells/well) stably expressing the *F. hepatica* (A, B) or the human taurine transporter (C, D) were incubated for 10 min in the presence of $0.1 \mu\text{M}$ $[^3\text{H}]$ taurine and of the indicated concentrations of unlabeled taurine and of GES. Data represent means $\pm$ S.D. from three independent experiments carried out in duplicate. The solid lines in panels A and C were drawn by fitting the data to a monophasic inhibition curve. In panels, B and D, the data shown in panels A and C, respectively, were replotted in Dixon plots to visualize the non-competitive and the competitive inhibitory action of GES by the intersecting lines (B) and the parallel lines (D), respectively.

Detection of the *F. hepatica* taurine transporter by immunoblotting

We raised a rabbit polyclonal antiserum against the N-terminus of the *F. hepatica* taurine transporter using a purified fusion protein comprising maltose-binding protein (MBP) and the N-terminus (i.e., residues R²-R⁵⁶) as the immunogen. We also generated the fusion of

glutathione-S-transferase (GST) and the N-terminus of the *F. hepatica* taurine transporter. The purified GST-fusion protein was bound to GSH-sepharose 4B, which allowed for the enrichment of the taurine transporter-specific antibodies by affinity purification. The resulting antibody preparation was tested on cell lysates prepared from untransfected HEK293 cells and from cells expressing either a YFP-tagged version of the *F. hepatica* taurine transporter or the non-tagged protein (Fig. 8A). Consistent with the multiple glycosylation sites of the *F. hepatica* taurine transporter (*cf.* Fig. 2), the antibody detected several bands of ~70 kDa and ~95 kDa in cell lysates containing the un-tagged (Fig. 8A, lane 2) and the YFP-tagged version (Fig. 8A, lane 3), respectively. These immunoreactive bands were not seen in lysates prepared from untransfected cells (Fig. 8A, lane 1) and from cells expressing human taurine transporter (data not shown). As an additional control, we also used an antibody against GFP for detection of the YFP-tagged transporter (Fig. 8B): the immunoreactive bands, which were visualized by the anti-GFP antibody, were identical to those detected by the antibody raised against the N-terminus (*cf.* right-hand lanes in Fig. 8A and Fig 8B).

Homogenates were prepared from adult *F. hepatica*; in addition, we enriched for tegumental cell membranes by subjecting adult flukes to a freeze-thaw cycle combined with vigorous vortexing. This procedure allows for collecting the particulate material from the tegument from the adult flukes and for the substantial enrichment of surface markers [40–42]. Although the sensitivity of the immunoblot sufficed to detect the heterologously expressed transporter in 1 µg of cell lysate (Fig. 8C, lanes 2 and 3 for the untagged and YFP-tagged transporter respectively), it was not possible to detect any immunoreactive material of the expected molecular mass in lysates from adult flukes (Fig. 8C, lane 4). However, the preparation enriched in tegumental membranes contained immunoreactivity for the taurine transporter (Fig. 8C, lane 5).

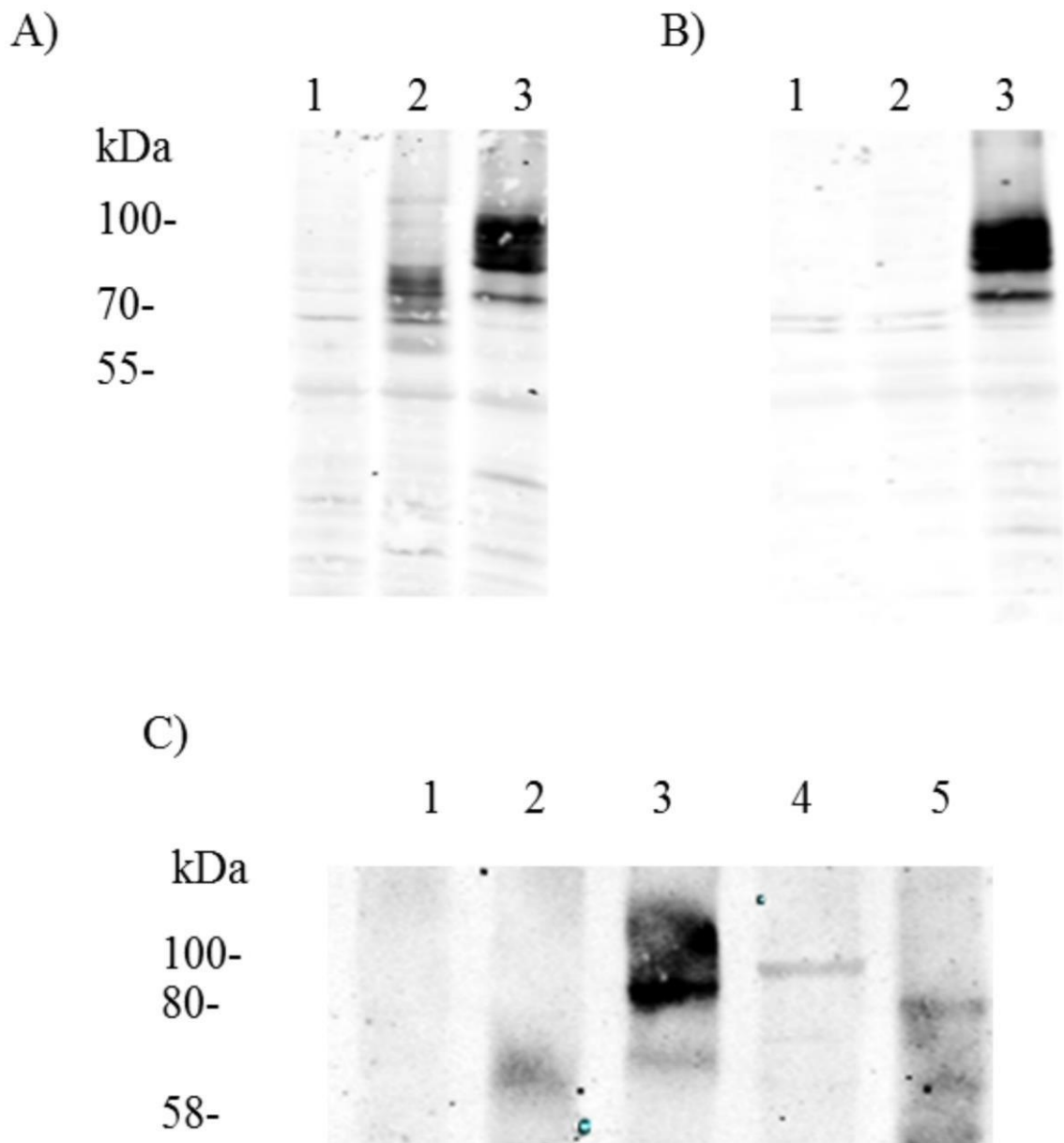


Fig. 8. Detection by immunoblotting of the *F. hepatica* taurine transporter after heterologous expression (A, B) and in lysates prepared from adult flukes (C). A&B: lysates (20 µg/lane) were prepared from untransfected HEK293 cells (lane 1), from HEK293 cells stably expressing the *F. hepatica* taurine transporter (lane 2) and a YFP-tagged version of the transporter (lane 3). After electrophoretic separation on a SDS-polyacrylamide gel, the proteins were transferred to a nitrocellulose membrane, which was probed with an affinity purified rabbit polyclonal antibody raised against the N-terminus (A) and an anti-GFP antibody (B). **C:** lysates (1 µg) prepared from untransfected HEK293 cells (lane 1) or from HEK293 expressing the untagged (lane 2) and the YFP-tagged *F. hepatica* taurine transporter (lane 3) were resolved by denaturing gel electrophoresis together with detergent extracts (40 µg) of adult fluke homogenates (lane 4) and of tegumental particulate material (lane 5). After transfer to nitrocellulose membranes, the immunoreactive bands were visualized as described for panels A & B. The immunoblots are representative of four independent experiments.

Survival of *F. hepatica*

Living adult *F. hepatica* can survive more than 14 days in Hédon-Fleig medium [43]. Accordingly, we placed adult flukes into the Hédon-Fleig medium. Under control conditions and in the presence of 50 μ M taurine or of 2 mM GES, all flukes were alive and motile for at least 6 h (Fig. 9A). In contrast, if flukes were exposed to a mixture of unconjugated bile acids, i.e. cholic acid, chenodeoxycholic acid and deoxycholic acid as found in bovine bile [44], flukes were progressively killed such that their median survival was about 3 hours (open triangles in Fig. 9A). We exposed a total number of 35 flukes to bile acids for 4 h, which was lethal for about two thirds (second bar in Fig. 9B). Flukes were protected against the deleterious effect of bile acids by the presence of 50 μ M taurine in the medium, which afforded a statistically significant protection: survival increased to about 80% in the presence of bile acids and taurine (third bar in Fig. 9B). This protective effect was abolished, if GES was also present in the medium, with only 44% surviving (fourth bar in Fig. 9B).

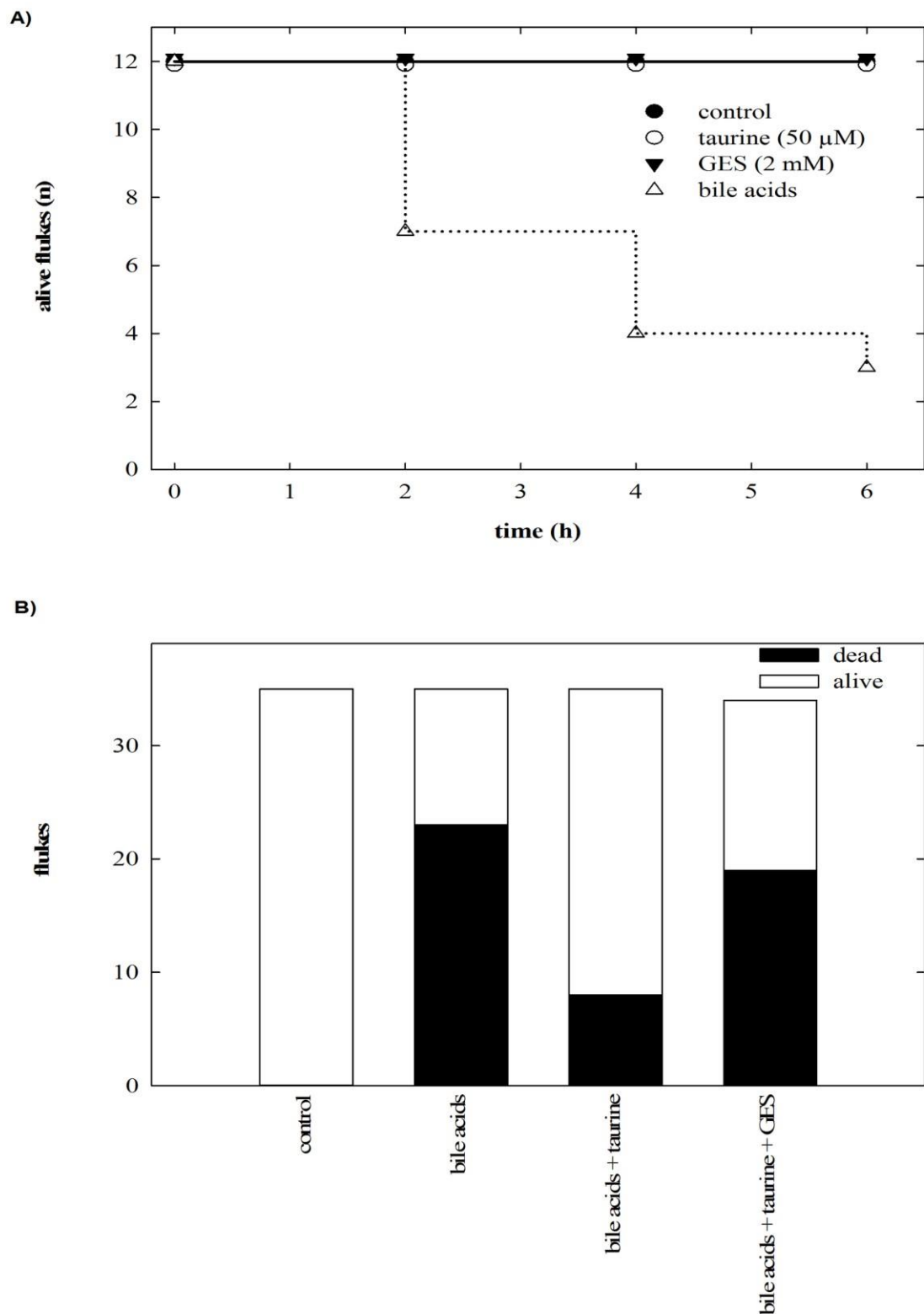


Fig 9. Survival of adult *F. hepatica* in bile acid-containing medium in the presence and absence of taurine. **A:** Adult flukes (12 flukes/condition in individual wells) were incubated at room temperature in Hédon Feig medium in the absence (control, closed circles) or presence of 50 μ M taurine (closed circle), of 2 mM GES (GES, closed triangle) or of bile acids (closed triangle, 5 mM cholic acid, 1.5 mM deoxycholic acid and 0.5 mM chenodeoxycholic acid). The number of dead flukes was recorded at the indicated time points. **B:** Adult flukes were incubated at room temperature in Hédon Feig medium in the absence (control) or presence of bile acids (5 mM cholic acid, 1.5 mM deoxycholic acid and 0.5 mM chenodeoxycholic acid). Where indicated, the medium contained, in addition, 50 μ M taurine or the combination of 50

μ M taurine and 2 mM GES (GES) for 4h. The number of dead flukes was recorded at the end of the incubation period. The data are from three independent experiments with 11 to 12 flukes/condition. Survival in the presence of bile acids and taurine differed significantly from that observed in the sole presence of bile acids ($p=0.0006$, Fisher's exact test) and in the presence of the combination of bile acids, taurine and GES ($p=0.0068$, Fisher's exact test), but there was no significant difference between survival in the presence of bile acids and of the combination of bile acids, taurine and GES ($p=0.464$). Thus, after Bonferroni correction for multiple testing (with $\alpha \leq 0.016$), taurine afforded a statistically significant protection against bile acids.

Discussion

The SLC6 transporter family has four branches: (i) the neurotransmitter transporters (i.e., transporters for dopamine, norepinephrine and serotonin), (ii) the glycine and proline transporters, (iii) the amino acid transporters SLC6A15 to SLC6A20 and (iv) the GABA transporter subfamily, which mediates the uptake of GABA, betaine, creatine and taurine [45]. The evolutionary history of this latter branch was traced by sequence comparisons and by examining chromosomal synteny [46]; the following sequence of events was proposed: gene duplication of the ancestral transporter gene gave rise to the GABA-transporter-1 (GAT-1/SLC6A1) and the creatine transporter-1 (CT1/SLC6A8). The taurine transporter (TauT/SLC6A6) is the third gene, which appeared during evolution, presumably by duplication of the creatine transporter-1. In chordates, GAT3 (SLC6A11) arose from TauT. GAT2/BGT1 (betaine/GABA-transporter-1, SLC6A12) and GAT4 (SLC6A12) were later additions during vertebrate evolution. Here we identified an SLC6 family member in *F. hepatica*. We classified this transporter as a taurine transporter based on both, its sequence homology with the other protostomial taurine transporter, for which the biochemical activity has been verified, i.e. the TauT of *Bathymodiolus septemdierum* [30] and an in-depth characterization of its biochemical specificity: our data unequivocally show that taurine is the preferred substrate of the transporter. In fact, the affinities of the alternative substrates β alanine and GABA were about 50- and 350-fold lower, respectively, than that of taurine. Taurine uptake was dependent on both, Na^+ and Cl^- . This was to be expected: with the notable

exception of SLC6A17, which is thought to function as a Na^+ -dependent vesicular amino acid transporter [47,48], all eukaryotic SLC6 transporters function as Na^+/Cl^- dependent plasma membrane transporters. The majority relies on a stoichiometry of 2 sodium ions and one chloride ion. This was also true for the *F. hepatica* taurine transporter: chloride enhanced transport velocity in a hyperbolic fashion, but sodium stimulated taurine uptake with a sigmoidal concentration-response curve resulting in a Hill-coefficient close to 2. This 2:1 stoichiometry is also consistent with the finding that the residues, which define the binding sites for the two sodium ions and the chloride ion are conserved in the *F. hepatica* taurine transporter.

Taken together, our observations showed that the *F. hepatica* taurine transporter differed from its human orthologue in several respects: (i) the chloride affinity of the *F. hepatica* taurine transporter was substantially higher than that of the human transporter. From a teleological perspective, this finding can be rationalized as an adaptation to the ionic composition of the bile: the chloride concentration in mammalian bile is substantially lower than that of plasma [49]. Thus, an affinity in the range of 12 mM assures that the fluke transporter operates at close to saturation of the chloride site regardless of the changes in ionic composition resulting from hormonal stimulation of bile flow [49]. (ii) Similarly, the K_M of the *F. hepatica* transporter for taurine was lower than that of the human orthologue. The concentration of taurine in human plasma is in the range of 50 μM [50]; thus the K_M of the human taurine transporter is close to the extracellular levels. Although taurine was originally identified in ox bile [51], the free concentration of free taurine in bile is - to the best of our knowledge - not known. We suspect that the higher affinity of the *F. hepatica* taurine transporter reflects an adaptation to the lower concentration of taurine in bile. (iii) The pharmacology of taurine transporters has not yet been explored in depth. In spite of the limited availability of inhibitors, our observations show that the *F. hepatica* transporter differs substantially from that of the human taurine transporter: GABA and β -alanine were about 3 and 5-fold less potent,

respectively, in inhibiting the *F. hepatica* than the human transporter. The affinity of GES for the *F. hepatica* was also lower. Importantly, GES was a non-competitive inhibitor of the *F. hepatica* transporter. The non-competitive mode of inhibition can be rationalized by taking into account that SLC6 transporters harbor two binding sites, namely the vestibular S2 site and substrate binding site proper, which is referred to as S1 site [45,52]. The non-competitive action may arise if GES binds preferentially to the S2 site: occupancy of the vestibular site and of the substrate binding site proper is not mutually exclusive and results in non-competitive inhibition [53]. At the very least, our observations justify the assumption that the taurine transporter of *F. hepatica* differs enough to allow for the development of selective specific inhibitors, which target the fluke transporter but not the mammalian orthologue.

The mechanism by which taurine accumulation protects *F. hepatica* from bile acid toxicity is not known. In mammals, taurine deficiency or genetic deletion of the taurine transporter results in pleiotropic effects, which culminate in retinal degeneration, liver and kidney disease, skeletal muscle wasting, etc. [54,55]. It is generally accepted that taurine is an osmolyte, which protects cells against various types of stress at least in part by stabilizing proteins against denaturation [56]. Bile acids in particular deoxycholic acid are chaotropic and promote unfolding of proteins [57]. Thus, it is plausible to posit that taurine accumulation in flukes is a safeguarding mechanism, which blunts bile acid toxicity. The alternative hypothesis is to assume that flukes use taurine as a substrate to conjugate free bile acids; the resulting conjugated bile acids are subsequently re-exported into the bile by an ABC-transporter. This hypothetical mechanism of detoxification requires at least two components, (i) a bile acid-CoA: amino acid N-acyltransferase and (ii) a bile salt export pump. In fact, adult *F. hepatica* express many ABC transporters: we identified 60 ABC transporters in a separate transcriptome analysis of adult flukes; several of these fell into the B family (unpublished observation), which also includes ABC-B11, the mammalian bile salt export pump (BSEP). Previously, a murine monoclonal antibody, which had been raised against a peptide derived from the first

nucleotide binding domain (Y⁷⁰⁵-K⁷¹⁸) of human ABC-B11, was used to immunoblot lysates of adult *F. hepatica*: an immunoreactive band was detected, albeit of only 80 kDa, which is half the size of the human orthologue [58]. Hence, it is not clear, if adult flukes express a bile salt export pump. In addition, we failed to find any evidence for the presence of a bile acid-CoA: amino acid N-acyltransferase by analyzing either the deposited genomic sequence [19,20] or the sequenced transcripts of adult flukes (unpublished observation). Regardless of the underlying mechanism, our observations suggest that the taurine transporter is essential for the survival of *F. hepatica* in hostile environments. The protective action of intracellular taurine is presumably the driving force for the early appearance of the taurine transporter during evolution [46]. We suspect that the hypothetical GABA-transporter-2 of *Clonorchis sinensis* is, in fact, a taurine transporter. The same is likely to be true for the other trematode SLC6 transporters in Fig. 3, i.e., *Opisthorchis viverrini* hypothetical protein, the *Schistosoma haematobium* GABA transporter-2 and the *Schistosoma mansoni* serotonin transporter. Hence, designing specific inhibitors may also be of interest to explore the role of these transporters in the biology of these parasites.

Materials and Methods

Reagents and chemicals

If not stated otherwise, cell culture plastic dishes and pipettes were from Sarstedt AG&Co., Nuembrecht, Germany, chemical and reagents including cell culture media were from Sigma Aldrich.

Collection and processing of adult flukes

Adult flukes were collected from the bile ducts of freshly slaughtered cattle in local abattoirs (Eschenau & Salzburg, Austria) and washed with phosphate-buffered saline (PBS; composition:

2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 4.3 mM Na₂HPO₄ x 2H₂O, pH 7.3) and either maintained in Hedon Fleig solution (120.7 mM NaCl, 4 mM KCl, 1.9 mM MgSO₄, 0.9 mM CaCl₂, 18.5 mM NaHCO₃, 10 mM HEPES, 15 mM D-glucose adjusted pH to 7.3) [43] or frozen in liquid nitrogen and stored at -80 °C.

PCR amplification of cDNA and cloning

Total RNA was isolated from freshly isolated adult flukes using Trizol® (Sigma Aldrich, Mannheim, Germany) Reverse transcription was performed using “Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany), using either gene specific primers or oligo dT primers. Starting from a previously deposited cDNA sequence 5’ and 3’ ends of the cDNA were identified via RACE (rapid amplification of cDNA ends) technology, using 5’/3’ RACE Kit, 2nd Generation ® (Roche Diagnostics GmbH, Mannheim). Recognition sites for XhoI and KpnI were added to the full-length cDNA of FhTauT by PCR. The resulting PCR product was cloned via XhoI and KpnI to peYFP-C1 (Takara Bio Europe, France) generating a transporter tagged with a yellow fluorescent protein at its N-terminus. To generate a non-tagged transporter, FhTauT was also cloned to pcDNA3.1 (Invitrogen, Carlsbad, USA). HsTauT was cloned in a similar way to peCFP-C1 via BamHI and HindIII thereby generating a transporter tagged with the cyan fluorescent protein. Generated sequences predict the were validated by Sanger sequencing (LGC Genomics, Berlin, Germany). The cDNA sequence of FhTauT was deposited at GenBank (NCBI, Bethesda, USA) under the accession number: MG674191.

Bioinformatic analysis

The partial initial sequence of Fh TauT mRNA was obtained from the the database published by [18] and full length mRNA was obtained applying RACE techniques (details are written at PCR

amplification of cDNA and cloning of M&M). The genomic sequence is obtained from the database WormBase (<https://parasite.wormbase.org>) [19] and is based on the published *Fasciola hepatica* genome (PRJEB6687) [59].

Exon-intron boundaries of the final extended FhTauT sequence were detected using the splign web interface (<https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi>) [60]. The visualization of exons and introns organization in the genome were done by Exon-Intron Graphic Maker (<http://wormweb.org/exonintron>).

Orthologous sequences were identified using the basic local alignment tool (BLAST) web interface provided by NCBI [61]. Sequence alignment and phylogenetic analysis was performed using MEGA 7 [31] Multiple Sequence Comparison by Log-Expectation (MUSCLE [62]) and Clustal W [63] respectively. The results were displayed using ENDscript (<http://endscript.ibcp.fr/>) [64]. The following sequences were included in the analysis: *Clonorchis sinensis*, accession no: GAA52609.1; *Opisthorchis viverrini*, XP_009175490.1; *Schistosoma mansoni*, XP_018646439.1; *Schistosoma haematobium*, XP_012792810.1; *Bathymodiolus septemdiurnum*, BAF95543.1; *Drosophila melanogaster*, NP_651930.2; *H. sapiens*, P31645.1; *H.sapiens*, NP_003034.2; *H. sapiens* NP_057699.2.

The NetNGlyc 1.0 Server (www.cbs.dtu.dk) was used to search for putative N-glycosylation sites in the final sequence of FhTauT [65]. We used the crystal structure available, data of Hs SERT was derived from Coleman et al. (PDB ID:5I6X, DOI: 10.2210/pdb5i6x/pdb) to compare the protein structure with FhTauT and other transporters [21].

Cell line generation and uptake measurements

Human embryonic kidney-293 (HEK293 (ATCC® CRL-1573, LGC standards Wesel, Germany)) cells, were grown in Dulbecco`s modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FCS Nuaille, France), 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C

and with 5% CO₂ in a humidified incubator. Cells were transfected with plasmids encoding fluorescently tagged or untagged versions of *F. hepatica* (Fh TauT) and Homo sapiens (Hs TauT) taurine transporter using Jet Prime transfection reagent (Polyplustransfection, France). To generate monoclonal cell lines stably expressing the transporters, transfected cells were subjected to and selected with the concentration of 0.2 mg/ml geneticin (G418) for 10 days. Surviving cells forming colonies were separated and analyzed for membrane localization of transporters and transport of taurine. After the selection process cells were kept at 50 µg/ml G418 to keep the selection pressure.

For saturation uptake experiments, cells were plated the day before the experiment at a density of about 50,000 cells/well onto poly (D-lysine)-coated 48-well culture plates (CytoOne, USA). On the day of the experiment, cells were washed with prewarmed Krebs-HEPES buffer twice (KHB) (120 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 20 mM glucose, 10 mM HEPES, pH 7.3). Then, cells were incubated in KHB containing tracer amounts (0.1 µM) of [³H] taurine, (19.1 Ci/mmol) (Perkin Elmer, USA) together with increasing concentrations of non-labeled taurine 10 min. The reaction was terminated after 10 minutes by removing the medium followed by rapid rinsing of cells with ice-cold assay buffer. Subsequently, cells were lysed with 0.5 ml of 1% Sodium Dodecyl Sulfate (SDS) and transferred into scintillation vials for liquid scintillation counting. Non-specific uptake was determined by incubating cells in the presence of the blocker β-alanine (100 mM) before and during the experiment and subtracted from uptake values. Data were fit to a Michaelis-Menten equation.

Chloride-free KHB was prepared to check chloride dependence of both FhTauT and HsTauT using acetate salts instead of chloride salts. Likewise, NaCl was replaced by choline chloride to check sodium dependence. Uptake experiments were performed as described above using 0.1 µM [³H] taurine in sodium, and chloride-free KHP modified buffers respectively, mixed with increasing amounts of NaCl containing KHB thereby varying Na⁺ and respectively Cl⁻ concentration from 0-120mM.

The inhibition experiments were performed using the same procedure as in the uptake experiments except only tritiated taurine was used (0.1 μ M) with the increasing concentrations of non-labeled inhibitors.

Confocal microscopy

For confocal imaging, HEK293 cells stably expressing YFP-Fh TauT and CFP-Hs TauT were seeded onto poly-D-lysine-coated glass-bottomed chambers 24 h prior to the experiment. Cells were imaged using a 60x oil immersion objective (Plan Apo VC, Nikon, Austria) on a confocal laser scanning microscope (A1R+, Nikon, Vienna, Austria). The fluorophores YFP and CFP were excited using a 488 nm and 403.5 nm laser line, respectively, at 1-5% of maximal intensity. Emission of CFP and of YFP was detected with a standard PMT (photomultiplier tube) detector equipped with a 435 nm emission filter (50 nm bandpass) and a GaAsP detector equipped with a 525 nm filter (50 nm bandpass). The cell membrane was visualized by incubating the cells in a trypan blue solution (0.05 %) for 5 min. Fluorescence of trypan blue was excited using a 561.9 nm laser line, the emission was detected with a GaAsP detector with a 595 nm filter (50 nm bandpass).

Sample preparation and Western blotting

Freshly isolated flukes were washed twice with PBS. Afterwards, flukes were homogenized at 4°C in HME buffer (10 mM HEPES, 1 mM MgCl₂, 0.1 mM EDTA, and pH 7.4) using an UltraTurrax Dispersing Instrument (Janke&Kunkel, IKA-WERK, Germany). The sheared flukes underwent two freeze/thaw cycles in liquid nitrogen. After repeated sonication, the lysate was centrifuged at 14 krpm for 15 minutes at 4°C. The pellet was resuspended in 5 ml of buffer (1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 10 mM Tris-Cl (pH 7.4)) supplemented with one tablet protease inhibitor per 20 ml (cOmplete Protease Inhibitor Cocktail, Roche)), incubated overnight on a rocking platform and centrifuged at 14 krpm at 4°C for 10 min. Supernatants from cell lysates were mixed with Laemmli buffer (0.1% 2Mercaptoethanol, 0.01%

Bromophenol blue, 10% Glycerol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8) and used for SDS page gel electrophoresis.

For *Fasciola* tegument preparation we used a “freeze-thaw and vortex” method described by Roberts et al. [41] applying minor modifications. About 5 g adult flukes (corresponding to 20 flukes) were flash frozen in liquid nitrogen and then thawed on ice in 5 ml of cold RPMI-1640 including protease inhibitors as described above. By vigorously shaking for 1 min the tegument was detached from the bodies of the flukes, and the supernatant was filtered through a metal sieve. The denuded bodies were pelleted by centrifugation at 1000g for 30 min at 4°C. The resulted pellet was resuspended in HME buffer, and freeze-thaw was done for twice. The produced sample was sonicated thrice. The sample was centrifuged at 40,000g for 2 hours at 4°C. The pellet was solubilized with Laemmli buffer.

Proteins were separated via SDS-PAGE electrophoresis and transferred from the gel to a nitrocellulose membrane. Membranes were blocked in Tris-buffered saline (TBS) containing 0.1-0.5% Tween 20 and 3% bovine serum albumin (BSA) or 3% skimmed milk. For detection, the purified antibody against N-terminus was used at a dilution from 1:100 to 1:50. The following commercial antibodies were used-rabbit polyclonal anti-GFP (Ab290 Abcam; 1/5000), anti-rabbit IRDye 680RD or anti-rabbit IRDye 800CW (LiCOR Biosciences Fluorescence signal on membranes was detected by Licor Odyssey CLx, (Imaging System).

Generation of polyclonal antibodies

Two rabbits were immunized with a fusion protein construct consisting of maltose binding protein, and the amino-terminus of FhTauT (RQEFSPSKSRTELAATSSIHVPFEVKDECSIIPVSSSLANKKEVEKSPREQWKR). The immunization was carried out at the Medical University of Vienna, Div. Laboratory Animal Science and Genetics, Himberg, Austria. Short: The antigen solutions for the immunization of one rabbit were prepared by emulsifying 100 µg MBP fusion protein in 750 µl 1x PBS (aqueous solution) and 1 ml (in)complete Freund's adjuvant (oleaginous solution). The emulsions were

kept at 4 °C, and the immunization was performed within 1 h. Antibodies were affinity purified out of serum from immunized rabbits. For this 6-His and glutathion-Stransferase (GST) was fused to the same N-terminal peptide of FhTauT as mentioned above and expressed in *E. coli* BL21. Subsequently, the protein eluted by cOmplete™ His-Tag Purification Resin (Sigma Aldrich, Mannheim, Germany) under denaturing conditions and urea content was reduced from 8 M to 5 M with a saline solution. To optimize the coupling to Affi-gel 10 (BioRad, California, USA), eluates were pH adjusted based on their isoelectric point titrating either NaOH or HCl. To block free binding sites of the Affi-gel 10, 100 µl 1 M ethanolamine pH 8.0 per ml Affi-gel 10 suspension were added for 1 h at room temperature. Antibodies were eluted with alkaline elution buffer (pH 11.5). 1 ml fractions were collected in Eppendorf tubes prefilled with 100 µl acidic neutralization buffer (pH 2.45). Protein-containing fractions were pooled, and the protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce, ThermoFisher Scientific).

Survival assay

F. hepatica was isolated from the bile ducts of cows slaughtered at Alpenrind GmbH (Salzburg, Austria). Flukes were kept in modified Hedon Fleig's solution at 37 °C and 5% CO₂ in a humidified atmosphere. Before the experiment, flukes were maintained at 37 °C for three days after extraction to clear them from bile acids. Living flukes (judged by observing movement after gently tapping them with metal forceps) were transferred into flasks containing either Hedon Fleig's Solution alone or Hedon Fleig's Solution supplemented with combinations of bile acids (5 mM Cholic Acid (CA), 1.5 mM deoxycholic acid (DCA) and 0.2 mM chenodeoxycholic acid (CDC)), 50µM Taurine and 2 mM Guanidinoethyl sulfonate (GES). Survival of flukes was determined again after four hours of treatment.

Statistics

Data are expressed as arithmetic means $\pm$ S.E. Statistics Nonlinear regression analysis was carried out for uptake assays to determine the K_m values using the Sigma Plot software program (version 12.5; Systat Software, Chicago, IL, USA) or GraphPad Prism software (version 5.04, GraphPad Software, La Jolla California, USA). Hill coefficients for Na^+ and Cl^- dependent experiments were obtained from the sigmoidal curves using the softwares as noted above.

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3. Discussion

Fasciolosis is a severe production-limiting disease that infects livestock animals. It develops after livestock become infected with the pathogenic *F. hepatica* and *F. gigantica* parasitic flatworms and is the source of an estimated US \$2 billion of losses within the agricultural sector on an annual basis (Spithill *et al*, 1999). The majority of losses that are experienced in the livestock sector result from mortality, secondary bacterial infections, reductions in milk and meat production, condemnation of livers at slaughter, and costly anthelmintic treatments (Garcia *et al*, 2008; McKenna *et al*, 2002; Roberts *et al*, 2006). It is very difficult to control *F. hepatica* because of its complex life cycle, which comprises both an intermediate snail host and a definitive mammalian host. During the snail host lifecycle, the parasite can multiply asexually. As such, when an egg is released from the definitive host, it can subsequently progress to produce thousands of infective metacercariae (Roberts *et al*, 2006). It is possible to control fasciolosis to some degree through a combination of effective pasture control practices and the use of drugs to reduce the production of metacercariae in snail host populations. However, in addition to being impractical, these approaches tend to be nothing more than short-term solutions (Crossland, 1976; Armour *et al*, 1970). The most common method of controlling infection with *F. hepatica* is by treating the infected livestock with anthelmintics in the form of flukicides. The most common compound in use to treat liver fluke infections is Triclabendazole (TCBZ) (Brennan *et al*, 2007). Since its introduction in the early 1980s, TCBZ has been proven to be effective against the parasite at all stages of its lifecycle while alternative flukicides are typically only effective when the parasite reaches the adult stage (Fairweather & Boray, 1999).

Cases of TCBZ-resistant fluke populations of flukicides were first reported in Australia (Overend & Bowen, 1995), and additional cases have later been identified throughout the world (Mitchell *et al*, 1998; Moll *et al*, 2000; Alvarez-Sánchez *et al*, 2006). In light of the

emergence of parasites that are resistant to the current treatment methods, there is a requirement to develop novel drugs or drug combinations that are effective against fascioliasis.

3.1. Characterization of SLC6 transporters and its role in survival of *F. hepatica*

The SLC6 transporter family is comprised of four branches:

1. the neurotransmitter transporters, which transport the monoamines serotonin, dopamine, and norepinephrine;
2. the glycine and proline transporters;
3. the amino acid transporters SLC6A15 to SLC6A20; and
4. the GABA-transporter subfamily, which translocates betaine, GABA, taurine, and creatine (Bröer & Gether, 2012).

The evolutionary history of the GABA-transporter subfamily was previously examined by studying sequence comparisons and considering chromosomal synteny (Kinjo *et al*, 2013). As a result, the following sequence of events has been proposed: gene duplication of the ancestral transporter gene resulted in the development of the GABA-transporter-1 (GAT-1/SLC6A1) and the creatine transporter-1 (CT1/SLC6A8). The taurine transporter (TauT/SLC6A6) is the third gene to emerge during evolution. This is believed to have resulted from the duplication of the creatine transporter-1. In chordates, GAT3 (SLC6A11) arose from TauT. GAT2/BGT1 (betaine/GABA-transporter-1, SLC6A12) and GAT4 (SLC6A12) were later additions during the evolution of vertebrate. In the current research, we identified an SLC6 family member in *F. hepatica*. This transporter was classified as a putative taurine transporter according to its sequence homology with the protostomial taurine transporter, for which the biochemical activity has been verified, i.e., the TauT of *Bathymodiolus septemdierum* (Inoue *et al*, 2008). We also

performed a comprehensive characterization of its biochemical specificity, and the results conclusively demonstrated that the preferred substrate of the transporter is taurine.

Indeed, the affinities of the GABA and β -alanine alternative substrates were approximately 350- and 50-fold lower, respectively, than those of taurine. The level of taurine uptake was contingent on both Na^+ and Cl^- . This result was consistent with expectations: excluding SLC6A17, which is believed to operate as a Na^+ -dependent vesicular amino acid transporter (Parra *et al*, 2008; Zaia & Reimer, 2009), all eukaryotic SLC6 transporters act as Na^+/Cl^- -dependent plasma membrane transporters. The majority of SLC6 transporters rely on a stoichiometry of one chloride ion and two sodium ions. This was also the case with the *F. hepatica* taurine transporter: while chloride increased transport velocity in a hyperbolic manner, sodium-stimulated taurine uptake with a sigmoidal concentration-response curve resulted in a Hill-coefficient that was close to two. The observed 2:1 stoichiometry is consistent with the finding that the residues, which delineate the binding locations for the chloride ion and the two sodium ions are preserved in the *F. hepatica* taurine transporter.

In combination, the results of the current study demonstrate that the *F. hepatica* taurine transporter differs from its human orthologue in three main regards:

1. The *F. hepatica* taurine transporter exhibited a significantly higher chloride affinity than that of the human transporter. From a teleological point of view, it is possible to rationalize this finding as a result of evolutionary adaptation: the concentration of chloride found in mammalian bile is substantially lower than that found in plasma (Wheeler & Ramos, 1960). Thus, the *F. hepatica* taurine transporter presumably evolved to operate effectively when faced with the ionic composition of the bile: with an affinity in the range of 12 mM, the occupancy of the chloride site is predicted to be close to saturation,

irrespective of any modifications in the ionic concentration that results from the hormonal stimulus of bile flow (Wheeler & Ramos, 1960).

2. The K_M of the *F. hepatica* transporter for taurine was less than that of the human orthologue. Human plasma contains a concentration of taurine in the range of 50 μM (Lepage *et al*, 1997); as such, the K_M of the human taurine transporter is near to the extracellular levels. While taurine was first detected in ox bile (Tiedemann & Gmelin, 1827), to the best of our knowledge, the free concentration of free taurine in bile is unknown. There is a possibility that the higher affinity of the *F. hepatica* taurine transporter is indicative of adaptation to the lower level of taurine that is present in the bile.
3. The pharmacology of taurine transporters has not yet been subject to an in-depth exploration. Hence, the availability of inhibitors is limited. Nevertheless, the observations in the current study indicate that there is a significant difference in the pharmacology of the *F. hepatica* transporter and that of the human taurine transporter. Specifically, the alternative substrates β -alanine and GABA were approximately five- and threefold less potent, respectively, at inhibiting the *F. hepatica* than the human transporter. The affinity of the *F. hepatica* transporter for guanidinoethyl sulfonate was also lower. Notably, guanidinoethyl sulfonate acted as a non-competitive inhibitor of the *F. hepatica* transporter. This finding can be accounted for by considering the fact that SLC6 transporters have two binding sites: the vestibular S2 site and the substrate binding site proper (S1) (Bröer & Gether, 2012; Zhu *et al*, 2016). A non-competitive action may arise if GES binds preferentially to the S2 site: occupancy of the vestibular site and the substrate binding site proper is not mutually exclusive and gives rise to non-competitive inhibition (Sarker *et al*, 2010). Non-competitive inhibition can also arise if substrate and inhibitor preferentially bind to different states in the conformational cycle of the transporter. A case in point is the plant-derived alkaloid ibogaine, which traps the serotonin transporter in the inward-facing state and therefore acts as a non-competitive inhibitor of serotonin uptake

(Jacobs *et al*, 2007; Bulling *et al*, 2012). However, conformational trapping is predicted to result in mutually exclusive binding of substrate and inhibitor. Regardless of the underlying mechanistic details, the results of the current study are at the very least consistent with the hypothesis that the *F. hepatica* taurine transporter differs sufficiently to allow for the development of selective, specific inhibitors, which target the fluke transporter but do not inhibit the mammalian orthologue.

There is a lack of understanding of the process by which the accumulation of taurine protects *F. hepatica* from bile acid toxicity. In mammals, pleiotropic effects develop in response to taurine deficiency or the genetic deletion of the taurine transporters. These pleiotropic effects can lead to kidney and liver disease, skeletal muscle wasting, retinal degeneration, etc. (Warskulat *et al*, 2007; Ito *et al*, 2014). Taurine is an osmolyte, which protects cells against some types of stress by stabilizing proteins against denaturation (Kumar, 2009). Some bile acids, especially deoxycholic acid, are chaotropic and promote protein unfolding (Cremers *et al*, 2014). It is conceivable that the accumulation of taurine safeguards the flukes against the toxicity of bile acid by preventing protein denaturation. As an alternative hypothesis, flukes may be assumed to employ taurine as a substrate to conjugate free bile acids, which are re-exported in conjugated form by an ABC-transporter into the bile. For this hypothesis to be true, the detoxification process will require at least two elements: (i) a bile salt export pump and (ii) a bile acid-CoA: amino acid N-acyltransferase. Adult *F. hepatica* does express a range of ABC transporters; in fact, during our transcriptome analysis of adult flukes, we identified 60 ABC-transporters. Several of these fell into the B family (unpublished observation), which - in mammals - includes the bile salt export pump (BSEP/ABC-B11). In a previous study, lysates of adult *F. hepatica* were immunoblotted with a murine monoclonal antibody, which had been raised against a peptide derived from the first nucleotide binding domain (Y⁷⁰⁵-K⁷¹⁸) of human ABC-B11. An immunoreactive band of 80 kDa was detected, which was only half the size of the human orthologue (Kumkate *et al*, 2008). Hence, it is not clear, whether adult flukes express a bile salt export pump. We did also not find any evidence for the presence of a bile acid-CoA: amino acid

N-acyltransferase by analyzing either the sequenced transcripts of the adult flukes (unpublished observation) or the deposited genomic sequence (Howe *et al*, 2016a, 2017). Irrespective of the underlying mechanism, our findings indicate that the *F. hepatica* requires the taurine transporter to survive in hostile environments. Intracellular taurine has a protective action that is believed to act as the driving force behind the early appearance of the taurine transporter during the process of evolution (Kinjo *et al*, 2013). We hypothesize that the hypothetical GABA-transporter-2 of *Clonorchis sinensis* and the other trematode SLC6 transporters depicted in Fig. 3, that is, the *Schistosoma haematobium* GABA-transporter-2, the *Schistosoma mansoni* serotonin transporter, and the *Opisthorchis viverrini* hypothetical protein are also taurine transporters. The development of specific inhibitors is likely to be useful because they allow exploring the physiological role of these transporters in the life cycle of these parasites.

3.2. Conclusions and future prospects

More than 55% of the loss of farm animals in Europe can be attributed to helminth parasites. Developing methods for effective parasite control is likely to have a major long-term impact on the livestock industry (Nieuwhof & Bishop, 2005; Murphy *et al*, 2006; Morgan *et al*, 2013). The majority of parasitic diseases observed in farm animals in Europe result from infestations by gastrointestinal nematodes of sheep (*Teladorsagia circumcincta*, *Haemonchus contortus*) and cattle (*Cooperia oncophora*, *Ostertagia ostertagi*), by the liver fluke *Fasciola hepatica* and the lungworm *Dictyocaulus viviparus*. These parasites have a direct impact on the productivity of livestock by negatively impacting the quality of wool and milk production, growth rates, and fertility. During seasonal fluke outbreaks, it is not uncommon for infection to result in the death of large numbers of animals (Piedrafita *et al*, 2010). The currently approved anthelmintic drugs were developed during the second half of the 20th century with praziquane (approved in 1980) and ivermectin (approved in 1987) as the latest additions. The vast majority of anthelmintic these drugs were discovered by serendipity rather than by a target-based approach. The current thesis provided a

proof-of-concept for targeting transmembrane transport in a parasitic worm. There are several arguments to support the claim that transmembrane transporters represent good candidate drug targets: (i) parasites are by definition not auxotrophic, they depend on the host to provide nutrients. (ii) Most, if not all, nutrients require transmembrane transporters to reach the cytosol. (iii) The evolutionary distance between mammals and parasites is large. Hence, transporters are likely to have diverged to an extent, which can be exploited to achieve pharmacological specificity. Thus, it is safe to predict that a systematic study of parasite transporters is likely to yield useful drugs.



4. Materials and Methods

4.1. Ethics Statement

Rabbits were immunized at the “Department für Biomedizinische Forschung, Medical University of Vienna”. This institution holds a permission (BMWF-66.009/0266-II/3b/2013) by the Austrian Ministry of Science to immunize rabbits according to §26 Austrian Animal Testing Law of 2012 (TVG 2012 (BUNDESGESETZBLATT & FÜR DIE REPUBLIK ÖSTERREICH, 2012)). All efforts were made to minimize animal suffering and to reduce the number of animals used.

4.2. Reagents and chemicals

If not stated otherwise, cell culture plastic dishes and pipettes were from Sarstedt AG&Co., Nuembrecht, Germany, chemical and reagents including cell culture media were from Sigma Aldrich.

4.3. Collection and processing of adult flukes

According to European Regulation (EC 854/2004) (European Parliament and of the Council, 2004), the livers of slaughtered cattle have to be inspected by veterinarians for a possible infestation of animals with parasites using visual, palpation and incision inspection (Dupuy *et al*, 2013). Based on this surveillance, livers associated with liver flukes are discarded together with other abnormalities (Dupuy *et al*, 2013). Before this, we inspected these livers for the presence of *F. hepatica*. Adult flukes were collected from the bile ducts of freshly slaughtered cattle in local abattoirs (Eschenau & Salzburg, Austria) and washed with phosphate-buffered saline (PBS; composition: 2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 4.3 mM Na₂HPO₄ x 2H₂O, pH 7.3) and either maintained in Hedon Fleig solution (120.7 mM NaCl, 4 mM KCl, 1.9 mM MgSO₄, 0.9 mM CaCl₂, 18.5 mM NaHCO₃, 10

mM HEPES, 15 mM D-glucose adjusted pH to 7.3) (Dawes, 1954) or frozen in liquid nitrogen and stored at -80 °C.

4.4. PCR amplification of cDNA and cloning

Total RNA was isolated from freshly isolated adult flukes using Trizol® (Sigma Aldrich, Mannheim, Germany) Reverse transcription was performed using “Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany), using either gene specific primers or oligo dT primers. Starting from a previously deposited cDNA sequence 5’ and 3’ ends of the cDNA were identified via RACE (rapid amplification of cDNA ends) technology, using 5’/3’ RACE Kit, 2nd Generation ® (Roche Diagnostics GmbH, Mannheim). Recognition sites for XhoI and KpnI were added to the full-length cDNA of FhTauT by PCR. The resulting PCR product was cloned via XhoI and KpnI to peYFP-C1 (Takara Bio Europe, France) generating a transporter tagged with a yellow fluorescent protein at its N-terminus. To generate a non-tagged transporter, FhTauT was also cloned into pcDNA3.1 (Invitrogen, Carlsbad, USA). HsTauT was cloned in a similar way into peCFP-C1 via the BamHI and HindIII restriction sites thereby generating a transporter tagged with the cyan fluorescent protein. The integrity of the constructs was verified by Sanger sequencing (LGC Genomics, Berlin, Germany). The cDNA sequence of FhTauT was deposited at GenBank (NCBI, Bethesda, USA) under the accession number MG674191.

4.5. Bioinformatic analysis

The partial initial sequence of Fh TauT mRNA was obtained from the database published by (Young *et al*, 2010); a full length cDNA was obtained by the RACE technique (for details see section 4.3.). The genomic sequence was obtained from the database WormBase (<https://parasite.wormbase.org>) (Howe *et al*, 2016b), which hosts the published *Fasciola hepatica* genome (PRJEB6687) (Cwiklinski *et al*, 2015).

Exon-intron boundaries of the final extended FhTauT sequence were detected using the splign web interface (<https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi>) (Kapustin *et al*, 2008). The visualization of exons and introns organization in the genome were done by Exon-Intron Graphic Maker (<http://wormweb.org/exonintron>).

Orthologous sequences were identified using the basic local alignment search tool (BLAST) web interface provided by NCBI (Altschul *et al*, 1990). Sequence alignment and phylogenetic analysis was performed using MEGA 7 (Kumar *et al*, 2016), Multiple Sequence Comparison by Log-Expectation (MUSCLE (Edgar, 2004)) and Clustal W (Thompson *et al*, 1994) respectively. The results were displayed using ENDscript (<http://endscript.ibcp.fr/>) (Robert & Gouet, 2014). The following sequences were included in the analysis: *Clonorchis sinensis*, accession no: GAA52609.1; *Opisthorchis viverrini*, XP_009175490.1; *Schistosoma mansoni*, XP_018646439.1; *Schistosoma haematobium*, XP_012792810.1; *Bathymodiolus septemdierum*, BAF95543.1; *Drosophila melanogaster*, NP_651930.2; *H. sapiens*, P31645.1; *H.sapiens*, NP_003034.2; *H. sapiens* NP_057699.2.

The NetNGlyc 1.0 Server (www.cbs.dtu.dk) was used to search for putative N-glycosylation sites after conceptual translation of the FhTauT cDNA (Gupta & Brunak, 2002). We used the crystal structure of human SERT (Coleman *et al*, 2016) PDB ID:5I6X, DOI: 10.2210/pdb5i6x/pdb) to compare the protein structure with FhTauT and other transporters.

4.6. Cell line generation and uptake measurements

Human embryonic kidney-293 (HEK293) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FCS), 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C and with 5% CO₂ in a humidified incubator. Cells were transfected with plasmids encoding fluorescently tagged or untagged versions of *F. hepatica* (Fh TauT) and Homo sapiens (Hs TauT) taurine transporter using Jet Prime transfection reagent (Polyplus-transfection, France). To generate monoclonal cell lines stably expressing the transporters, transfected cells were

subjected to selection in the presence of 0.2 mg/ml geneticin (G418) for 10 days. Surviving cells forming colonies were separated and analyzed for membrane localization of transporters and transport of taurine. After the selection process cells were maintained in culture medium containing 50 µg/ml G418 to keep the selection pressure.

For saturation uptake experiments, cells were plated the day before the experiment at a density of about 50,000 cells/well onto poly (D-lysine)-coated 48-well culture plates (CytoOne, USA). On the day of the experiment, cells were washed with prewarmed Krebs-HEPES buffer twice (KHB) containing 120 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 20 mM glucose, 10 mM HEPES, pH 7.3. Then cells were incubated in KHB containing tracer amounts (0.1 µM) of [³H]taurine, (19.1 Ci/mmol) (Perkin Elmer, USA) together with increasing concentrations of non-labeled taurine. The reaction was terminated after 10 min by removing the medium followed by rapid rinsing of cells with ice-cold assay buffer. Subsequently, cells were lysed with 0.5 ml of 1% sodium dodecylsulfate (SDS) and transferred into scintillation vials for liquid scintillation counting. Non-specific uptake was determined by incubating cells in the presence of the blocker β-alanine (100 mM) before and during the experiment and subtracted from uptake values. Data were fitted to the Michaelis-Menten equation.

Chloride-free KHB was prepared to determine the chloride-dependence of both FhTauT and HsTauT using acetate salts instead of chloride salts. Likewise, NaCl was replaced by choline chloride to determine the sodium dependence. Uptake experiments were performed as described above using 0.1 µM [³H]taurine in sodium- or chloride-free buffer, respectively, mixed with increasing amounts of NaCl containing KHB thereby varying the Na⁺ or Cl⁻ concentration from 0-120mM.

The inhibition experiments were performed using the same procedure as described for the uptake experiments except that only tritiated taurine was used (0.1µM) with increasing concentrations of non-labeled inhibitors.

4.7. Confocal microscopy

For confocal imaging, HEK293 cells stably expressing YFP-Fh TauT and CFP-Hs TauT were seeded onto poly-D-lysine-coated glass-bottom chambers 24 h prior to the experiment. Cells were imaged using a 60x oil immersion objective (Plan Apo VC, Nikon, Austria) on a confocal laser scanning microscope (A1R+, Nikon, Vienna, Austria). The fluorophores YFP and CFP were excited using a 488 nm and 403.5 nm laser line, respectively, at 1-5% of maximal intensity. Emission of CFP and of YFP was detected with a standard PMT (photomultiplier tube) detector equipped with a 435 nm emission filter (50 nm bandpass) and a GaAsP detector equipped with a 525 nm filter (50 nm bandpass). The cell membrane was visualized by incubating the cells in a trypan blue solution (0.05 %) for 5 min. Fluorescence of trypan blue was excited using a 561.9 nm laser line, the emission was detected with a GaAsP detector with a 595 nm filter (50 nm bandpass).

4.8. Sample preparation and Western blotting

Freshly isolated flukes were washed twice with PBS. Thereafter, flukes were homogenized at 4°C in HME buffer (10 mM HEPES, 1 mM MgCl₂, 0.1 mM EDTA, and pH 7.4) using an Ultra-Turrax Dispersing Instrument (Janke&Kunkel, IKA-WERK, Germany). The sheared flukes underwent two freeze/thaw cycles in liquid nitrogen. After repeated sonication, the lysate was centrifuged at 16,100 g for 15 minutes at 4°C. The pellet was resuspended in 5 ml of buffer (1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 10 mM Tris-Cl (pH 7.4.) supplemented with one tablet protease inhibitor per 20 ml (cOmplete™ Protease Inhibitor Cocktail, Roche), incubated overnight on a rocking platform and centrifuged at 16,100 g at 4°C for 10 min. Supernatants from cell lysates were mixed with Laemmli buffer (0.1% 2-mercaptoethanol, 0.01% bromophenol blue, 10% glycerol, 2% SDS, 62.5 mM Tris.HCl, pH 6.8) and used for SDS page gel electrophoresis.

For Fasciola tegument preparation we used a “freeze-thaw and vortex” method described by Roberts et al. (Roberts *et al*, 1983) with minor modifications. About 5 g adult flukes (corresponding to 20 flukes) were flash frozen in liquid nitrogen and then thawed on ice in 5 ml of cold RPMI-1640

including protease inhibitors as described above. By vigorously shaking for 1 min the tegument was detached from the bodies of the flukes, and the supernatant was filtered through a metal sieve. The denuded bodies were pelleted by centrifugation at 1000 g for 30 min at 4°C. The resulting pellet was resuspended in HME buffer and subjected to two freeze-thaw cycles. The produced suspension sample was sonicated thrice. The sample was centrifuged at 40,000 g for 2 hours at 4°C. The pellet was solubilized with Laemmli buffer.

Proteins were separated via SDS-PAGE electrophoresis and transferred from the gel to a nitrocellulose membrane. Membranes were blocked with 3% bovine serum albumin (BSA) or with 3% skimmed milk in Tris-buffered saline (TBS) containing 0.1-0.5% Tween 20. For detection, the purified antibody against N-terminus was used at a dilution from 1:100 to 1:50. The following commercial antibodies were used: rabbit polyclonal anti-GFP (Ab290 Abcam; 1/5000), anti-rabbit IRDye 680RD or anti-rabbit IRDye 800CW (LiCOR Biosciences). The fluorescence signal on membrane was detected in a Licor Odyssey CLx Imaging System (LiCOR Biosciences).

4.9. Generation of polyclonal antibodies

Two rabbits were immunized with a fusion protein construct consisting of maltose binding protein and the amino-terminus of FhTauT (RQEFSPSKSRTELAATSSIHPVFEVKDECSIIPVSSSLANKKEVEKSPREQWKR). The immunization was carried out at the Medical University of Vienna, Div. Laboratory Animal Science and Genetics, Himberg, Austria. The antigen solutions for the immunization of one rabbit were prepared by emulsifying 100 µg MBP fusion protein in 750 µl PBS (aqueous solution) with 1 ml complete Freund's adjuvant (oleaginous solution). The emulsions were kept at 4 °C, and the immunization was performed within 1 h. Booster injections were done at biweekly intervals with the antigen emulsified in incomplete Freund's adjuvant. Antibodies were affinity-purified from the serum from immunized rabbits. For this purpose, a construct was generated, which fused the sequence encoding the N-terminus of FhTauT (see above) to a hexa-histidine tagged glutathion-S-transferase (GST). *E. coli* BL21DE3 were transformed with the resulting expression plasmid, the protein was

expressed and purified from bacterial lysates using cOmplete™ His-Tag Purification Resin (Sigma Aldrich, Mannheim, Germany) under denaturing conditions. The urea concentration was reduced from 8 M to 5 M with a saline solution. To optimize the coupling to Affi-gel 10 (BioRad, California, USA), eluates were pH adjusted based on their isoelectric point by titrating with either NaOH or HCl. To block free binding sites of the Affi-gel 10, 1 M ethanolamine, pH 8.0, per (0.1 ml/ml Affi-gel 10 suspension) was added for 1 h at room temperature. Bound antibodies were eluted with alkaline elution buffer (pH 11.5). Fractions (1 mL) were collected in Eppendorf tubes prefilled with 100 µl acidic neutralization buffer (pH 2.45). Protein-containing fractions were pooled, and the protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce, ThermoFisher Scientific).

4.10. *F. hepatica* survival assay

Adult flukes was isolated from the bile ducts of cattle slaughtered at Alpenrind GmbH (Salzburg, Austria). Flukes were kept in modified Hedon Fleig's solution at 37 °C and 5% CO₂ in a humidified atmosphere. Before the experiment, flukes were maintained at 37 °C for three days after extraction to clear them from bile acids. Living flukes (judged by observing movement after gently tapping them with metal forceps) were transferred into flasks containing either Hedon Fleig's solution alone or Hedon Fleig's solution supplemented with a combination of bile acids (5 mM Cholic Acid (CA), 1.5 mM deoxycholic acid (DCA) and 0.2 mM chenodeoxycholic acid (CDC)), 50 µM taurine and 2 mM guanidinoethyl sulfonate (GES). Survival of flukes was determined again after 4 h of incubation.

4.11. Statistics

Data are expressed as arithmetic means ± S.E.M. Data from saturation and inhibition experiments were subjected to nonlinear least-squares regression to equations for a rectangular hyperbola and monophasic inhibition, respectively, using a Marquardt-Levenberg algorithm implemented in the Sigma Plot software program (version 12.5; Systat Software, Chicago, IL, USA) or in the GraphPad

Prism software (version 5.04, GraphPad Software, La Jolla California, USA). Slope factors (=Hill coefficients) for Na⁺ and Cl⁻ were obtained by fitting the data in a similar manner to the Hill equation (=three parameter logistic equation).

5. References

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